The role of endo-pectinases in pathogenicity as determined by the use of defined mutants of fungal plant parasites

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THE ROLE OF *ENDO*-PECTINASES IN PATHOGENICITY AS DETERMINED BY THE USE OF DEFINED MUTANTS OF FUNGAL PLANT PARASITES

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Abstract

Various evidence implicates *endo*-polygalacturonase (PG) produced by the necrotrophic pathogen of onion, *Botrytis allii*, is probably the fungal agent responsible for killing host cells in advance of the leading hyphae. Similarly *endo*-pectin lyase (PL) produced by the vascular parasite *Verticillium albo-atrum* (VAA) has been identified as a pathogenicity determinant. VAA and *B. allii* produced multiple forms of PG and PL whose synthesis varied with the carbon source on which they were grown. The major PG and PL of VAA have molecular weights (MW) of 37 500 and 50 000 daltons respectively and isoelectric points (pI's) of 6.8 and 10.6. VAA produced over 20 PG isozymes ranging from pI 3-10 and at least 6 isozymes of PL (pI 4-10.6). *B. allii* produce only a single form of *exo*-PL (MW, 26 000; pI 7.6), and at least 8 acidic PG isozymes. These enzymes are not constitutive but are induced by the substrate polymer, either when in purified form or as a component of isolated host cell walls. Both of the *B. allii* enzymes are subject to catabolite repression. The pectinases of VAA and *B. allii* are products of *de novo* protein synthesis, as induced production was inhibited by cyclohexamide.

PG and PL production coincided with lesion development on onion infected with *B. allii*. The PL and the major *endo*-PG isozyme (pI 5.4) were isolated from lesions 72 h after inoculation. Exposure of onion epidermal tissue to these enzymes showed that the *exo*-PL does not macerate or cause cell death and ion leakage. Conversely, *endo*-PG rapidly caused death of host cells. This evidence is discussed in relation to suppression of host defence.

Induced mutagenesis was performed to determine the rôle of PG and PL in disease by attempting to establish a correlation between reduced

pathogenicity and/or virulence, with altered pectinase production in pectinase-deficient mutants. Two solid pectic media were devised on which chemically-induced mutants lacking either PG or PL could be detected by their inability to degrade the pectic substrate. No mutants of *B. allii* or any specific PG-deficient VAA mutants were isolated. The reasons for this are discussed in relation to isozyme frequency. However, three PL-deficient mutants of VAA were isolated. They were identical to the +Type in morphology, growth rate and sporulation. Enzyme synthesis was critically determined in liquid and solid culture and PL levels were shown to be *c* 3, 9 and 43 % of the +Type. One mutant, 24d produced most of the PL isozymes but at reduced levels. A second mutant, 34i produced only basal levels of PG and PL and was additionally incapable of assimilating and/or metabolising the uronide degradation products from pectic polymers. The third mutant proved to be a secretory mutant and released lower levels of PG, PL, cellulase (C_1), leucyl arylamidase, galactosidase and glucosidase.

The abilities of pectinase-deficient mutants to infect host plants and cause symptoms was evaluated and compared to the +Type isolate. Comparative studies of symptom formation and quantitative assessments of vascular colonisation in tomatoes showed that symptoms were less severe or appeared later in plants infected with the mutants. However, each isolate, apart from the secretory mutant C23, colonised plants to levels comparable to +Type. This implies that the pectinases, particularly *endo*-PL, are virulence factors although they are probably not determinants of pathogenicity, because colonisation can proceed in their absence.

The potential for obtaining and using defined mutants with altered synthesis, regulation or secretion is discussed.

Abbreviations

Å	Angstrom
c	*circa*
°C	degree(s) centigrade
cm	centimeter(s)
CMC	carboxymethyl cellulose
conc.	concentration
CWDE	cell wall-degrading enzymes
CR	catabolite repression
CTAB	cetyltrimethyl ammonium bromide
d	day(s)
DAPI	4,6-diamidino-2-phenylindole
d.H_2O	distilled water
diam.	diameter
Ech	*Erwinia chrysanthemi*
EMS	ethylmethane sulphonate
g	gram(s)
GALA	saturated galacturonic acid
h	hour(s)
IEF	isoelectric focusing
K_{av}	diffusion coefficient
KC	kilocycle(s)
KV	kilovolt(s)
KW	kilowatt(s)
M	mole(s) or molar
mA	milliamp(s)
µg	microgram(s)
mg	milligram(s)
µl	microlitre(s)
ml	millitre(s)

mm	millimetre(s)
µmol	micromole(s) or micromolar
MW	molecular weight
MI	maceration index
min	min(s)
MX	medium X
NAPP	sodium polypectate
nm	nanometre(s)
NTG	*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine
OD	optical density
PAG	polyacrylamide gel
PAGE	polyacrylamide gel electrophoresis.
PG	polygalacturonase
PGL	polygalacturonate lyase
pI	isoelectric point
PL	pectin lyase
PME	pectin methyl esterase
ppm	parts per million
rpm	revolutions per minute
R_s	Stokes' radius
+Type	wild type
$t_{½}$	half-life
t_{50}	time to achieve 50 % reduction in viscosity
UGALA	unsaturated galacturonic acid
UV	ultraviolet
VAA	*Verticillium albo-atrum*
V_e	protein elution volume
V_o	Void volume
V_t	total volume
YEP	yeast extract peptone medium

Contents

Introduction

Endo-pectinases are unique amongst cell wall-degrading enzymes in that they have the ability to kill host cells. This probably reflects the central rôle which the polygalacturonide substrate maintains within the structure of the primary cell wall; its degradation alone results in loss of wall integrity and in some tissues leads to complete cell separation (Cooper, 1986b).

These destructive enzymes are produced by numerous facultative fungal and bacterial phytopathogens. Amongst necrotrophs, particularly the soft-rot bacteria, they would seem to have an important rôle during colonisation (Collmer *et al.*, 1982). The enzymes may be either determinants of pathogenicity and/or virulence and/or have importance in providing a nutrient carbon source for the parasites.

Pectinases have been found in diseased tissue at the onset of symptom formation (eg Cooper & Wood, 1980; Wijesundera *et al.*, 1984). Associated microscopic and chemical evidence has suggested their activity in the host (eg Bateman, 1976; McKeen, 1974). Pectinases may be directly responsible for symptom formation and cell killing or in contrast, these may result from indirect effects of polygalacturonan degradation as biologically active wall and cytoplasmic components are released (Bateman & Basham, 1976; Cooper, 1986b).

In spite of extensive studies of this kind, evidence with hemibiotrophs and necrotrophs remains largely circumstantial. Moreover, workers have frequently failed to detect *endo*-pectinases *in vivo*, (Cooper, 1986a).

A more pragmatic approach to the controversy of these potentially important enzymes, is through isolating pectinase-deficient mutants of pectolytic phytopathogens and subsequently testing their relative pathogenicity and virulence. In this way a direct correlation may be

established between the production of pectinases and disease. This rationale is currently being applied to the soft-rot bacterial diseases, caused by *Erwinia chrysanthemi* and *E. carotovora*, by modern gene cloning techniques (Collmer & Keen, 1986), but these techniques are only just developed for fungal pathogens. Previous attempts have been with fungi by conventional mutagenesis, but with only limited success, partly because of insufficient awareness of the complexity of pectinase types and production (eg Mann, 1962). However, this is a particularly good time to undertake such studies because of the improved understanding of the enzymes and their regulation and the many techniques now available for detecting and characterising the various specific pectinase isozymes produced by putative mutants (eg Bertheau *et al*, 1984).

The main objective of this work was to establish a rôle for polygalacturonase and pectin lyase in three diseases by selecting and characterising specific *endo*-pectinase-deficient mutants from the pathogenic +Type strains of three pectolytic phytopathogenic fungi with contrasting parasitic strategies. Such mutants should also further our understanding of the mechanisms of regulation and secretion of these enzymes both *in vitro* and *in vivo*.

Whereas bacterial pathogens colonise passively *via* wounds or natural openings, fungal pathogens such as the necrotroph of onion, *Botrytis allii*, the hemibiotrophic parasite of French bean, *Colletotrichum lindemuthianum*, and the vascular parasite of tomato *Verticillium albo-atrum* must actively penetrate the many cell walls that are encountered during colonisation. These barriers to infection are presumably overcome by the use of cell wall-degrading enzymes and particularly *endo*-pectinases. These enzymes may be of substantial importance to all three fungi, but could have contrasting rôles during pathogenesis. It

is hoped that the behaviour of the mutants during pathogenesis would reveal the true significance of the enzymes.

Although the emphasis of the work lay with the genetic approach outlined above, efforts were also made to further characterise the polygalacturonases and pectin lyases, and particularly the isozymes, to increase our insight into their importance to these parasites.

Literature Review

1. Composition and degradation of primary cell walls.

The first part of this section is a review of primary cell wall composition with special emphasis paid to the structural rôle of pectin. The remaining parts are concerned with enzymic cell wall-degradation and with particular reference to *endo*-pectinases and their ability to cause maceration and cell-killing. The properties of pectinases are also discussed.

1.1. Composition and structure of the primary cell wall.

Our understanding of cell wall structure and composition has advanced significantly during the last two decades with the onset of superior biochemical extraction and purification techniques in association with improved knowledge on the cleavage of specific polymeric bonds by reagents and well characterised cell wall-degrading enzymes (CWDE). Recent research on cell wall composition and structure has been reviewed by Albersheim (1976), Aspinall (1980), Darvill, Mc Neil, Albersheim & Delmer (1980a), Northcote (1972) and Selvendran (1985).

Primary cell walls which consist almost entirely of polysaccharides are laid down by undifferentiated plant cells during growth. These semi-rigid structures are sufficiently pliant to undergo elongation under osmotic pressure. After elongation the primary walls thicken by the integration of new polysaccharides, and in specialised cells, cutin (eg epidermis), lignin (eg xylem) and suberin (eg endodermis) are also deposited. Cell walls are by no means inert structures as they undergo substantial physiochemical changes and contain bound molecules which can have many diverse effects upon wall modification, during growth or under challenge from a pathogen (Cooper, 1981, 1984 and 1986b).

Most detailed studies on composition have employed cell walls from sycamore suspension cultures which provide a bulk of homogeneous

primary cell wall material with minimal contamination from secondary wall components. Nine main monosaccharides are found in the primary cell wall of the sycamore which has provided a model system for cell wall analysis (Albersheim, 1976). There are seven main hexoses in the cell wall polymers, three are stereoisomers, glucose, galactose and mannose. The other four are rhamnose and fucose which are both deoxyhexoses, and the carboxylated uronic acids galacturonic and glucuronic acids. The two main pentose sugars are arabinose and xylose. All of these monomers are combined in varying proportions to form the major structural polymers that surround the cellulose framework.

Cellulose is a homogeneous β-1,4, glucan comprising *c* 90 % of the glucose fraction in parenchyma tissue (Stevens & Selvendran, 1984d; Selvendran, 1985). The β-linked glucose chains have been shown, by X-ray crystallography, to be hydrogen bonded together as microfibrils that are composed of *c* 40 flat β-glucan ribbons. The microfibrils are separated by a continuous hydrated matrix of four major components: xyloglucan, arabinogalactan, rhamnogalacturonan and hydroxyproline-rich protein. Carbohydrate compositions have been determined by a combination of Gas chromatography (GC) and Mass spectroscopy of alditol acetate derivatives of monomers, cleaved from polymeric material by acid or enzymic hydrolysis (Talmadge, Keegstra, Bauer & Albersheim, 1973; Selvendran, 1985). Examples of the relative quantities of the components found in non-lignified cell walls are listed in Table 1 (from Selvendran, 1985). This reveals that the cell walls of apple parenchyma, immature onion tissues and pea cotyledons are rich in pectic substances, cellulose and 'hemicelluloses' (xyloglucans), whereas grass mesophyll cells are poor in pectic substances but rich in cellulose and hemicelluloses (acidic arabinoxylans). Many reports have confirmed that cell walls of members of the Gramineae contain

characteristically low levels of pectic substances. It is apparent that major differences can occur in the composition of the major non-cellulosic matrix polymers and this may determine the type of enzymic degradation undertaken by pathogens with different hosts; this will be considered more thoroughly in terms of wall structure and parasitic strategy in Literature Review 3.

Xyloglucans comprise xylose units attached to a backbone of β-linked glucose molecules in such a way that the glucose units are free to hydrogen bond with the β-glucans in cellulose fibrils. Xyloglucan molecules are glycosidically bound to arabinogalactan molecules and thereby link the fibrillar phase to the pectic fraction.

Arabinogalactans from various sources appear to have a 3-, 6- linked galactan backbone with arabinosyl side chains (Darvill *et al.*, 1980a). Arabinogalactan chains are thought to emerge from the xyloglucan coated surface of the cellulose fibrils as spokes are arrayed around the hub of a wheel. The ends of the spokes are bound to rhamnogalacturonan chains which run across the fibrils and essentially hold the cellulose-

Table 1. Carbohydrate compositions of cell walls from some non-lignified tissues.

Sugar *a*	Parenchyma Apples (1)	Immature Onions (2)	Mesophyll Grass (3)	Cotyledons Peas (4)
6-Deoxyhexose	2.0	1.1	1.0	2.0
Arabinose	12.3	1.5	13.9	48.0
Xylose	3.3	1.3	17.6	5.0
Mannose	4.3	0.7	0.0	≈0.0
Galactose	5.7	13.5	4.0	5.0
Glucose	22.7 *b*	31.2 *b*	57.2 *b*	24.0 *b*
Uronic acid	32.8 *c*	32.5 *c*	6.5 *c*	16.0 *c*

(1) Stevens & Selvendran (1984d); (2) Selvendran (1985); (3) Gordon, Morris, Rees, Smith & Thorn, (1977); (4) Brillouet & Carré (1983).

a Values are g anhydrosugar 100 g^{-1} cell wall material (dry weight).
b The bulk (≈95 %) of the glucose arises from cellulose.
c The bulk (≈95 %) of the uronic acid is D-galacturonic acid and is from pectic material.

xyloglucan-arabinogalacturonan in a highly cross-linked semi-rigid matrix (Albersheim, 1975).

Rhamnogalacturonan, which constitutes 16 % of the sycamore wall, consists of α-1,4-linked galacturonic acid (GALA) chains interspersed with 1,2-linked rhamnose (RHA) units that tend to interrupt the ordered chain (Darvill *et al.*, 1980a) causing it to buckle in a zig-zagged conformation (Talmadge *et al.*, 1973). RHA is not uniformly distributed but occurs in RHA-GALA-RHA units which alternate through the chain with homogalacturonan sequences of 8-12 residues, although longer stretches of galacturonan, (>25 units), do exist.

Although a great deal of information has emerged from studies with cultured sycamore cells, doubts have arisen as to the credibility of using such a model system for determining the composition and structure of parenchymatous tissue (Selvendran, 1985); eg suspension cells are virtually devoid of middle lamella and uronic acid levels in walls derived from cultures are low, whereas arabinose and hydroxyproline-rich protein levels tend to be high. The proposed primary cell wall model may be too simplistic in the light of recent information concerning the location and infra-structure of pectic polymers, xyloglucans and polysaccharide-protein-polyphenol complexes (O'Neill & Selvendran, 1980a; Redgwell & Selvendran; Selvendran & Bushnell unpublished results, cited in Selvendran, 1985; Stevens *et al.*, 1984d). The properties and importance of the pectic polymers described in the next section have recently been reviewed by Fogarty & Kelly, (1983); Morris (1986), Rees (1982) and Selvendran (1985).

The rhamnogalacturonan backbone is additionally bedecked with side chains of galactan, araban and xylan (Aspinall, 1980). Furthermore, the GALA units may be methylated or non-methylated; the degree of methylation determines the extent to which a rhamnogalacturonan

molecule will chelate with divalent ions, particularly Ca^{2+} (and Mg^{2+}). Ca^{2+} causes a characteristic gelation of unmethylated pectic polymers in solution (Pilnik & Voragen, 1970). The bulk of the pectin which is found in the middle lamella, tends to be highly esterified and side chains are short, often only a single residue; the chains are also interlinked with Ca^{2+} and possibly by ester bonds (Selvendran, 1985). Evidence obtained from middle lamella of immature onions showed that the polymers could be separated in the presence of a chelating agent which removed the Ca^{2+} ions (Redgwell & Selvendran unpublished results, cited in Selvendran, 1985). In contrast, the primary cell wall contains pectin that is highly branched and interlinked with glyco-proteins, proteoglycans and xyloglucans (as outlined above).

1.2. Degradation of extracted cell walls *in vitro* by pectinases.

The central rôle of pectic substances in primary wall strucure can be readily highlighted by incubating extracted cell wall material with purified *endo*-pectinases which rapidly solubilise the GALA components. Basham and Bateman (1975a) showed that *c* 65 % of the pectic fraction (Arabinan, RHA and GALA) was released from tobacco walls by a polygalacturonate lyase (PGL) of *Erwinia chrysanthemi*. Similarly the action of an *endo*-polygalacturonase (PG) from *Colletotrichum lindemuthianum* on sycamore walls released the neutral sugars, arabinose and galactose in addition to GALA (Talmadge *et al.*, 1973), which also proved that arabinogalactan is covalently linked to the rhamno-galacturonan chains. These two examples further emphasise the significance of the structural function of pectin within the wall.

Pectinases serve as wall-modifying enzymes and render the non-pectic polymers more vulnerable to decomposition by other CWDE (English, Maglothin, Keegstra & Albersheim, 1972; Karr & Albersheim, 1970). Thus

sycamore cell walls were resistant to the actions of *endo*-glucanase (Bauer *et al.*, 1973; Keegstra *et al.*, 1973) and protease (Keegstra *et al.*,1973) unless pre-treated with an *endo*-polygalacturonase of *Colletotrichum lindemuthianum*. This may be a special case as no evidence of such synergism has been found in other systems studied subsequently (Baker, Whalen & Bateman, 1977; Cooper, Rankin & Wood, 1978).

The galactan chains linked to rhamnogalacturonan are degraded by *endo*- and *exo*-galactanases and β-galactosidase whereas the degradation of the arabinan component is invariably achieved by *exo*-enzymes (Bauer *et al.*, 1973; Byrde & Willetts, 1977; Cooper *et al.*, 1978).

Many pathogenic and saprophytic bacteria and fungi produce enzymes capable of decomposing the hemicellulosic and cellulosic components of the primary cell wall. *Endo*-glucanases break down the xyloglucan chains into small fragments which can no longer bind to the cellulose fibrils. Organised cellulose fibrils are relatively resistant to enzymolysis but the β-glucan chains are gradually hydrolysed by cellulase complexes comprising *endo*-glucanase (C_x), to yield oligomeric intermediates, (Eriksson, 1977) which are immediately acted on by *exo*-glucanases (C_1) which release cellobiose. Cellobiose, which can act as a competitive inhibitor of cellulose degradation is split into its constituent two glucose units by the action of β-glucosidase. 'Hemicellulose' and cellulose degradation have been extensively reviewed by Enari (1983), Eriksson (1977), Bateman & Basham (1976) and Cooper (1983 & 1986b).

1.3. Maceration and cell killing.

Tissue degradation by *endo*-pectinases may result in cell separation or maceration which is invariably accompanied by cell death. However, the mechanism(s) by which plasma membranes are damaged in the presence of these enzymes remains unknown (Bateman & Basham, 1976). Although other

degradative enzymes such as cellulases, hemicellulases, phosphatases and proteases are sometimes present, maceration is a unique process of degradation by *endo*-pectinases and does not occur in their absence (Bateman, 1966; Mount, Bateman & Basham, 1970; Collmer & Keen, 1986; Stephens & Wood, 1974). Cell injury is characterised by loss of ability to retain neutral dyes, a rapid loss of ions and water and a failure to plasmolyse in hypertonic solutions. There is strong evidence to suggest that cell death results from membrane disruption (Hall & Wood, 1973). This is generally thought to involve pectic degradation and weakening of the wall so that it can no longer withstand the pressure of the protoplast. Bateman & Basham (1976b) studied the toxic effects of PGL on potato tuber tissue. Purified PGL from *E. chrysanthemi* solubilised 50 % of wall sugars from isolated tobacco and potato cell walls. The enzyme also caused rapid maceration of potato discs and uronide degradation products were detected as they were released. Cell injury was detected as a rapid loss of electrolyte (within 3 min) from treated potato discs and furthermore, ion loss and the release of wall sugars was proportional to PGL activity. Under conditions of plasmolysis, purified PGL did not cause membrane damage, but when the hypertonic mannitol solution was replaced with hypotonic K phosphate buffer rapid ion loss occurred, as the tissue underwent deplasmolysis. Thus, degradation by PGL resulted in membrane damage only when the tissue was deplasmolysed and the weakened primary walls were under osmotic pressure (Basham & Bateman, 1975b). This and other similar evidence supports the hypothesis that cell injury results directly from damage to the plant cell walls (Stephens & Wood, 1975).

However, electron micrographs of apple suspension cells treated with purified PL from *Monilinia fructigena* showed that leakage and disruption to the plasmalemma may be concomitant or may even precede

noticeable wall degradation (Keon, 1985). Although gross wall integrity apparently remained unaltered during the early stages, iron hydroxamate staining revealed that methylated pectin was rapidly lost from the walls simultaneously with a loss of vital staining ability. PL induced cellular injury was achieved without plamalemma rupturing or extensive wall disorganisation. Injury apparently involved a biochemical interaction between the cell wall and the protoplasts, rather than as the exclusive result of osmotic fragility (Hislop, Keon & Fielding, 1979; Keon, 1985). Furthermore, in bean hypocotyls infected with the necrotroph *Rhizoctonia solani*, the plasmalemma was seen to retreat from the damaged wall (Kenning & Hanchey, 1980) which is an unexpected response if protoplast bursting occurs as a result in the lack of strength in the cell wall.

Polysaccharides and polygalacturonan itself have been shown to be closely associated with the plasmalemma (Albersheim & Killias, 1963; Keon, 1982; Roland & Vian, 1971) and there is the possibility that degradation could lead to an unknown interaction to cause leakage. In view of the early loss of pectin from the walls of the apple suspension culture cells and the almost immediate loss of vital staining, it is possible to speculate that this directly involves pectin degradation.

Some evidence suggests that pectic fragments *per se* may be phytotoxic. Fragments released from sycamore and soybean walls by acid hydrolysis were judged to be toxic to plant cells, based on inhibition of protein synthesis and trans-membrane movement of amino acids (Fry, Darvill & Albersheim, 1983; Yamazaki, Fry, Darvill & Albersheim, 1983).

Alternatively, membrane damage may result from indirect effects of pectin degradation. Cell wall-bound enzymes released during infection may directly or indirectly damage the plasma membrane. Peroxidase activity leads to lignification and the formation of toxic free-radical

intermediates that could damage membrane structure or in contrast could lead to the inhibition of the polygalacturonidases (Byrde & Archer, 1977; see Literature Review 1.5.). H_2O_2 generated by glucose oxidase activity could directly damage the plasma membrane and lead to the formation of a variety of symptoms (Mussell & Strand, 1977; see Literature Review 3.). Proteases and phospholipases released from lysed cells could directly damage plasma-membranes in adjacent tissue (Bateman & Basham, 1976; Cooper, 1983). Attempts to establish involvement of these factors in cell killing have proved negative (Bateman & Basham, 1976). However, it is very difficult to establish the toxicicty of factors released during tissue degradation as they may be labile or readily detoxified when they pass through the wall towards the membrane, eg, superoxide free radicals are detoxified by superoxide dismutase (McCord, 1974).

Ion loss from separated plasmolysed suspension cultured cells may actually be unrelated to pectin degradation and may occur as a result of direct membrane breakdown by the action of proteases and phosphatases (Stephens & Wood, 1974). Increased phospholipase and protease activity has been found in tissue infected with a number of organisms, eg bean tissue infected with *Thielaviopsis basicola* and cucumber leaves infected with *Pseudomonas lachrymans* (Lumsden & Bateman, 1968; Keen, Williams & Walker, 1967b). However, increased permeability occurred in bean leaves infected with *Uromyces phaseoli* 2-4 days before an increase in phospholipase activity was detected (Hoppe & Heitefuss, 1974). Moreover there is little evidence supporting a rôle for pathogen produced proteases in membrane degradation (Bateman & Basham, 1976), and furthermore the proteinase factor in extracts of *P. lachrymans*-infected tissue remains unidentified.

Proteases and phosholipases are produced by these pathogens *in vitro*, but the significance of phospholipid and protein degradation has so far not been realised.

1.4. Pectin degradation and properties of *endo*-pectinases.

Consideration will now be given to the biochemical nature of rhamnogalacturonan degradation followed by a description of some of the properties of *endo*-polygalacturonide-degrading enzymes; some of these aspects are reviewed by Bateman & Basham (1976) Byrde & Archer (1977), Cooper (1983, 1984 and 1986b), Fogarty & Kelly (1983), Keon, Byrde & Cooper (1986), Mussell & Strand, (1977), Rexová-Benková & Markovic (1976) and Rombouts & Pilnick, (1980). The α-1,4-bonds between the GALA units are cleaved by hydrolytic or lytic action. Hydrolysis is catalysed by the *endo*-polygalacturonases (PG; EC 3.2.1.15) and lysis, by *endo*-pectin lyases (PL; EC 4.2.2.10) and *endo*-polygalacturonate lyases (PGL; EC 4.2.2.2); lytic cleavage involves the formation of an unsaturated bond between C_4 and C_5 by a *trans*elimination reaction. Rhamnogalacturonan that has 75-85 % methylated carboxyl groups is defined as a pectin; unmethylated molecules are known as pectic acid (Bateman & Millar, 1966) and these are often complexed with cations eg sodium and calcium polypectate. PG's and PGL's specifically degrade unmethylated polypectate. PG activity is optimal in acidic conditions whereas PGL and pectin lyase activity is favoured in alkaline conditions. Although PG may degrade pectin, activity can invariably be attributed to the incompleteness of substrate methylation which leaves it vulnerable to hydrolysis. PG's are often inhibited in the presence of Ca^{2+} ions both directly but also because of the ionic bonds that form between the unmethylated carboxyl groups on the rhamnogalacturonan chains which cause the substrate to gel and presumably renders the

substrate molecules inaccessible to the enzyme. In contrast pectin lyases degrade pectin more effectively than polypectate and often require the presence of Ca^{2+} ions as cofactors. Methoxy groups of pectin are removed by pectin methyl esterases (PME; EC 3.1.1.11), which may act in concert with PG or PGL. A PME/PGL complex is produced by *Clostridium multifermentans* which rapidly alternates between demethylation and depolymerisation of pectin (Sheiman, Macmillan, Miller & Chase, 1976). In general, fungi produce PG and PL and bacteria secrete PGL (Cooper, 1983).

The enzymes are further classified into two groups according to their mode of action (Bateman *et al.*, 1966). *Endo*-enzymes cleave the galacturonan molecules at random and *exo*-enzymes remove residues terminally from the chains. The basis for classification is determined by relating the extent of GALA cleavage in a pectic solution with the viscosity reduction. *Endo*-enzymes cause a 50 % decrease in relative viscosity by cleaving 1-2 % of the glycosidic bonds; *exo*-enzymes must cleave 20-40 % of the bonds to effect the same change in viscosity (Nasuno & Starr, 1968; Kelly & Fogarty, 1978). The efficiency of enzyme activity and the size of the products released may change as the substrate is degraded and modified. Chain length has a marked effect on the affinity (K_m), the rate of bond cleavage and breakdown of the enzyme-substrate complex (V_{max}, as measured by product formation) by *endo*-PGL of *Bacillus polymyxa*; degradation of tetra- and tri-galacturonic acids was significantly less efficient than of polypectate (Nagel & Anderson, 1965). The division is too limiting as many *endo* enzymes initially attack chains at random and then release monomers or oligomers. Such a *multiple* attack is exerted by PG's of *C. lindemuthianum* and *Verticillium albo-atrum* (Cooper *et al.*, 1978; Talmadge *et al.*, 1973). *Erwinia chrysanthemi* produces an enzyme that

expresses both *endo*-PL and *exo*-PG activity. The two enzyme activities which were inseparable by ion-exchange chromatography and isoelectric focusing would seem to provide a second example of a complex (Stack, Mount, Berman & Hubbard, 1980). *Endo*-glucanase (C_x; EC 3.2.1.4) and *exo*-glucanase (C_1; EC 3.2.1.91) activity is so closely associated it has been postulated that these cellulases act as a loose complex (Wood & McCrae, 1979).

Sclerotium rolfsii releases Rha from cell walls presumably as a result of the α-rhamnosidase which it produces. It is not known whether cleavage occurs between GALA-RHA or RHA-RHA links within the rhamnogalacturonan chains. The signficance of such enzymes that cleave internal RHA links remains unknown (Cooper, 1983; Reese, 1977).

1.5. Limitation of pectin degradation by molecular sieving, binding and inhibition of pectinases.

In association with a generally improved understanding of the nature of rhamnogalacturonan degradation there has been a significant increase in our knowledge of the biochemical properties of pectinases. The size, MW, charge and stability of pectic enzymes accompanied by information concerning their vulnerability to inhibitors could shed some light on their possible rôle and significance *in vivo*.

The ability of a CWDE to enter the matrix of the primary cell wall and middle lamella may in part be determined by its MW (and therefore the effective radius), (Cooper, 1983; Cowling, 1977). Estimates of the pore size of the primary cell wall mesh range from *c* 40 Å (equivalent to a globular protein of 17 000 daltons) to 70 Å (which would exclude molecules of >60 000 daltons) (Carpita, Sabularse, Montezinos & Delmer, 1979; Tepfer & Taylor, 1981). *Endo*-PG's and PL's produced by many pathogens are small *c* 30 000 daltons (Table 2) and in theory could freely diffuse through the host tissues. The influence of size of CWDE on

efficacy was shown by (Knee, Fielding, Archer & Laborda, 1975) when a PG of 75 000 daltons degraded apple walls to about half the extent of a smaller PG (37 000 daltons), but a PG of *Phytophthora infestans* (300 000 daltons, Table 2) failed to degrade the wall at all. The largest PG was presumably prevented from entering the cell walls which were estimated to exclude molecules of >100 000 daltons. The restriction of the large *P. infestans* PG may explain why initial invasion of potato tissue by this hemibiotroph is accompanied by only minimal wall damage (Cooper, 1983).

Once the enzyme-substrate complex has been established in the wall, the effects of pectic degradation would expose the matrix polymers to further attack from similar or larger enzymes.

An alternative mechanism to 'molecular sieving' as a constraint to degradation is by inactivation and binding of CWDE to the cell walls; this may be either specific or non-specific (Cooper, Wardman & Skelton, 1981). CWDE bind to wall proteins, whose presence or absence is dependent on the method of wall extraction. Examples of these proteins, which were formerly ionically bound to cell walls, have been isolated and shown to inhibit PG's *in vitro* (Albersheim & Anderson, 1971; Fielding, 1980). Inhibition is generally reversible under increased ionic concentration and appropriate pH. The isoelectric point (pI) of a CWDE and the pH will also determine its affinity to bind to cell walls. Specific inhibitors of PG have been isolated from *Pisum sativum*, *Phaseolus vulgaris* and many other species (Hoffman & Turner, 1984; Lafitte, Barthe, Montillet & Touzé, 1984).

Many *endo*-PG's and PL's are very basic (Table 2) and positively charged at physiological pH values, giving them a high affinity for the negatively charged carboxyl groups on de-esterified rhamnogalacturonan, eg the *endo* PL of *Fusarium oxysporum* f.sp. *pisi* which has a pI of 9.1

was almost entirely immobilised (but reversible by salt desorption) by extracted cell walls (Cooper, 1986b). Many pectic enzymes have pI's and pH optima at a similar value; therefore at optimal pH they remain only weakly charged and less restricted by ionic attraction or repulsion from the walls (Cooper, 1986b; Montgomery, 1982). In contrast binding may enhance the activity of certain CWDE. A model has been put forward to explain how the formation of free carboxyl groups by the action of PME leads to the attraction of other enzymes which then instigate the loosening of bonds within the cell walls prior to expansion (Ricard & Noat, 1986). There is some evidence to suggest that the formation of ionic bonds with the substrate by *endo*-pectinases produced by pathogens may be a pre-requisite to successful degradation (Cervone, Scala & Scala, 1978). The alkaline isozymes of *Erwinia chrysanthemi* are more destructive to plant tissue than the neutral isozymes (Garibaldi & Bateman, 1971). This is probably because they attack the pectic substrate (Collmer & Keen, 1986) in a more random manner, but the greater acivity could be partially due to an increased capacity for ionic binding to the acidic polygalacturonate substrate.

The activity of PG and PL on plant tissue is largely non-specific, ie, pectinases produced by pathogens readily degrade non-host as well as host tissue (Bateman & Basham, 1976). In contrast a degree of enzyme-tissue specificity is suggested by the PG produced by *Rhizoctonia fragariae*, which strongly adsorbed to strawberry tissue but not to non-host tissue (Cervone, Scala, Foresti, Cacace & Noviello, 1977). PG isozyme 3 produced by *Botryodiplodia theobromae* bound strongly to host tissue which it also failed to macerate. Binding was reversible, non-specific and ionic; the enzymes were not inhibited or absorbed by the tissue as activity was readily recovered by desorption (Arinze, 1985).

During tissue maceration polyphenol oxidases come into contact with their previously compartmentalised substrates, oxidising them to quinones. Components of this reaction inactivate CWDE and may be important in resistance to subsequent degradation. Apart from their ability to inhibit a range of CWDE (see Byrde & Archer, 1977; Friend, 1976 & 1980 for reviews) some of these compounds are also fungitoxic. Inhibitors of PG produced by phenolases in apples seem to be involved in resistance to *S. fructigena* (Byrde, Fielding & Williams, 1960). *Penicillium expansum* on the other hand prevents tissue browning by inhibiting the host phenolase (Byrde *et al*, 1960; Byrde & Archer, 1977).

Experiments for testing pectinase susceptibility to phenolic inhibitors have often been performed over very long periods and with non-physiological concentrations of either, enzyme or inhibitor (Patil & Dimond, 1961; Bateman & Basham, 1976). Inhibitory phenols have also been chosen that are actually absent from the host plant (Byrde *et al.*, 1977). The end products of phenol oxidation may be relatively less important to inhibition than the intermediates, therefore, studies *in vitro* should be carried out with phenols that are being actively oxidised (Bateman & Basham, 1976). Inactivation of CWDE is mainly due to hydrogen bonding of the phenols to the enzyme protein although free radicals produced during oxidation may react with their NH_2 and SH side groups (Pierpoint, 1966).

Table 2. Examples of molecular weight, isoelectric point and calcium requirement of pectinases produced by some fungal and bacterial pathogens.

Organism	Enzyme	MW (daltons)	pI	Reference
E. chrysanthemi	*exo*-PG	67 000	8.3	1
E. chrysanthemi	*endo*-PGL	38 000 (isozymes c,g & f)		2
B. cinerea	*endo*-PG I	34 000	7.3	
	endo-PG II	56 000	7.6	3
C. lindemuthianum	*endo*-PL I	23 000	8.2	
	endo-PL II	28 500	9.7	4
Phoma medicaginis f.sp. *pinodella*	*endo*-PL	29 500 a	7.9	5
Phytophthora infestans	*endo*-PG	200 000	-	6
F. oxysporum f. sp. *lycopersici*	*endo*-PG	36 000	7.0	7
Verticillium dahliae	*endo*-PG	29 000	10.8 *b*	8

1) Collmer, Whalen, Beer & Bateman (1982); 2) Collmer, Schoedel, Roeder, Ried & Rissler (1985); 3) Marcus & Schejter (1983); 4) Wijesundera, Bailey & Byrde (1984); 5) Plumbley & Pitt (1979); 6) Knee, Fielding, Archer & Laborda (1975); 7) Strand, Rechtoris & Mussell (1976); 8) Wang & Keen (1970).

a The enzyme was also purified as a tetramer of MW 118 000 that also had a pI of 7.9.
b Theoretical value; Mussell & Strouse (1972) gave an experimental value of pI 9.7.

1.6. Frequency, detection and composition of PG and PL isozymes.

Improvements in electrophoresis and isoelectric focusing (IEF) techniques have revealed the presence of multiple forms of pectinases produced by all the bacterial and fungal pathogens so far examined. Traditional column IEF remains an essential step for preparing semi-pure isozymes and may even free non-active crude enzyme extracts (taken

Table 3. The number of pectinase isozymes resolved by preparative IEF (column) and PAGE IEF from tissue and culture extracts of some bacterial and fungal pathogens.

Organism	Source		IEF method		No. of isozymes		Ref.
	culture fluid *a*	tissue extract *b*	column *c*	PAGE *d*	PG	PL	
E. chrysanthemi	+		+		-	2 (PGL)	1
E. chrysanthemi	+			+	1	12	2
				+		14 (PGL)	3
Botrytis allii	+			+	4	-	4
B. cinerea	+		+		2	-	
	+			+	7-16	-	5
B. cinerea		+	+		2-4	-	6
C. lindemuthianum	+		+		1	2	
		+	+		0	1	7
V. albo-atrum	+		+		4 *d*	2	8
V. albo-atrum	+			+	9 *e*	-	9

1) Garibaldi & Bateman (1971); 2) Collmer, Schoedel, Roeder, Ried & Rissler (1985); 3) Bertheau, Madgidi-Hervan, Kotoujansky, Nguyen-The, Andro & Coleno (1984); 4) Kritzman, Chet & Gilan (1981); 5) Magro, Di Lenna, Marciano & Pallavivini (1980); 6) Di Lenna & Fielding (1983); 7) Wijesundera, Bailey & Byrde (1984); 8) Cooper, Rankin & Wood (1978); 9) Mohan & Ride (1984).

a Cultures contained either cell walls or pectin; isozymes produced by induced synthesis.
b Extracts from infected host tissue.
c Broad range IEF performed in a sucrose gradient (pH 3-10).
d PG isozymes resolved by narrow range IEF (pH 5-8).
e 9 bands isozymes were produced in cell wall culture; 5 isozymes were produced in sucrose by basal synthesis.

from infected tissue) from bound protein inhibitors (Fielding, 1981). The superior resolving power of polyacrylamide gel electrophoresis (PAGE) IEF and rapid detection by activity staining has revealed the presence of many more isozymes in culture filtrates of several pathogens than were previously resolved by column IEF, eg *Verticillium albo-atrum* (VAA) and *B. cinerea* (Table 3). PAGE IEF activity staining methods have also been adapted for detection of numerous glycanases such as xylanases and cellulases (MacKenzie & Williams, 1984). Crude pectinase zymogram patterns produced in slab gels containing pectin have been used to establish 11 distinct groups of 140 *Rhizoctonia* species and to distinguish between species of *Botrytis* and *Sclerotinia* (Cruickshank, 1983a & 1983b; Sweetingham, Cruickshank & Wong, 1986). Although a useful taxonomic tool, the resolution of column IEF is inferior to that obtained by PAGE IEF. PGL isozyme patterns from *E. chrysanthemi* culture filtrates have been resolved (Table 3) by applying ultrathin layers of polypectate/agarose gel, supported on plastic film, to PAGE IEF gels containing the focused enzymes. The isozymes were visualised with the cationic stain ruthenium red which precipitated the undegraded substrate to leave 'windows' of enzyme activity in the overlay (Ried & Collmer, 1985; Bertheau, Madgidi-Hervan, Kotoujansky, Nguyen-The, Andro & Coleno, 1984).

Using a simpler activity staining technique (Lisker & Retig, 1974) by overlaying the gel with a viscous solution of sodium polypectate followed by ruthenium red staining, Mohan & Ride (1984) resolved a broad range of PG isozymes produced by 13 hop isolates of VAA whereas conventional column IEF revealed only 4 peaks of PG activity (Cooper *et al.*, 1978). Discrepancies in total numbers of isozymes detected by the two methods are commonplace (Table 3). The gel IEF methods have the advantages of being quick, economical (24 samples can be resolved on a

PAG as opposed to only one in a column) and sensitive; as little as 1 ng or even 1 pg of enzyme protein may be detected (Ried & Collmer, 1985; MacKenzie & Williams, 1984); furthermore activity losses due to extended periods of dialysis that are necessary to remove carrier ampholines are also avoided.

The term isozyme refers to forms of an enzyme showing similar activity that are produced by a species as a result of more than one structural gene. Isozymes may be remnants of evolutionary change or present as progressive adaptations to a changing environment (Literature Review 3.). Complicated zymogram patterns may be misleading and may be generated by the separation techniques themselves. Different bands may not reflect the products of individual structural genes and may actually result from post-translational modifications to the protein structure (Collmer et al., 1985; Gianazza & Rignetti, 1980). Spurious bands can be readily distinguished from actual allelic products by two dimensional focusing of the gels (Ried & Collmer, 1985).

Slight differences in isoelectric point may reflect subtle changes in tertiary structure by the addition of a prosthetic group such as a carbohydrate molecule. The large variations in pI exhibited by many isozymes probably reflect more fundamental differences in primary structure as determined by their peptide composition and particularly in the ratio of acidic and basic amino acids, eg a PG of *V. dahliae* with a pI of 10.8 (Wang & Keen, 1970) contained more of the cationic, basic amino acids arginine and lysine and generally less of the anionic, acidic amino acids asparagine and glutamine than a PG of *Aspergillus niger* (Rexanová-Berková & Slezarik, 1968). Large differences in the pI's of two *isozymes* produced by a single organism are also likely to parallel discrepancies in primary structures eg, of the 4 isozymes of *B. allii* detected by Kritzman *et al.* (1981) that had

pI's of 5.3, 7.2, 7.9 and 8.1, isozymes 5.3 and 8.1 probably have quite dissimilar compositions and therefore are products of different loci. The position and the amount of charged amino acids present on the enzyme surface may well determine the type of enzyme-substrate complex produced or affect the efficiency of its formation. The presence of lysine residues on the surface of a PG could well be involved with substrate attachment (Keon, *et al.*, 1986). Rexanová-Berková & Markovic and McClendon (1970) postulated the presence of 3-5 or even 7 attachment subsites along the galacturonan chain for different PG's. The high lysine content of many PG's produced by necrotrophs may be explained by a need to bind to the substrate before degradation ensues (Cervone *et al.*, 1978; Keon *et al.*, 1986; see Literature Review 1.5.). Differential binding, inhibition and substrate preference of the isozymes produced may confer a greater flexibility on the pathogens for successful parasitism in a variety of tissues and hosts (Literature Review 3.) and possibly in alternative saprotrophic existences.

2. Regulation of synthesis of *endo*-pectinases.

This section is a review of the processes that control endo-pectinase production in bacteria and fungi, although, certain details concerning the regulation of other CWDE will be also be used to elaborate the discussion (see Cooper & Wood, 1973 and 1975; Cooper, 1977; 1983 and 1986b; Collmer *et al.*, 1982b; Enari, 1983; Fogarty & Kelly, 1983 for reviews). The mechanisms of induction, catabolite repression (CR) and secretion will be described with particular reference to recent genetic evidence provided by induced specific mutants (see reviews by Cooper, 1977, 1986a; Collmer *et al.*, 1982b; Collmer & Keen, 1986; Montenecourt, Schamhart & Eveleigh, 1979). A note on sugar transport and metabolism will also be included. The final part of this section will be a summary of the conditions and methods used to select defined mutants of bacteria and fungi with altered capacity to produce extracellular CWDE.

2.1. Induction and catabolite repression.

The model for the regulation of gene expression is still largely based on the *lac*-operon which controls synthesis of intracellular β-galactosidase in *E.coli* (Jacob & Monod, 1961; reviewed by Stanier, Adelberg & Ingraham, 1976). An alternative regulation system for an *extracellular* CWDE may be provided by the PGL of *E. chrysanthemi* (Ech) or *E. carotovora* (Collmer *et al.*, 1982b). As yet, no separate model has been proposed for gene control in eukaryotes and thus, enzyme synthesis is presumed to be regulated by a mechanism similar to that of the *lac*-operon of *E. coli*.

Our understanding of the processes of induction, repression and secretion has only advanced through a careful consideration of culture conditions. Unfortunately much of the work on regulation of CWDE production has involved long-term trials in unrestricted batch cultures

under unfavourable conditions, in which aspects such as the effects of catabolite repression (CR), pH, growth rate and autolysis have been ignored (Cooper, 1977).

Production of most polysaccharidases by bacteria and fungi is under the dual control of induction and catabolite repression (CR) (Magasanik, 1961). Production of an inducible CWDE depends on the presence of a potential inducer that is usually derived from the polymer substrate for which that particular enzyme is active. Induction follows, providing the amount of inducer does not exceed levels that will impose CR. Cooper & Wood (1975) demonstrated that synthesis of CWDE's produced by *V. albo-atrum* in inorganic salts culture, was specifically induced by the monomer predominant in each enzyme's substrate. This was evident only when inducers were supplied at rates which prevented CR; this was achieved by using diffusion capsules (Pirt, 1971) containing concentrated solutions of sugars which diffused out at a constant rate, dependent on the internal sugar concentration. Synthesis of *endo*-PG and *endo*-PL was almost entirely repressed when inducers were present in slight excess of requirements for growth; in contrast arabinase and β-galactosidase production were only partially reduced. Xylanase and arabinase were induced by restrictive supplies of xylose and arabinose whereas PG and PL were induced by GALA. The induction of pectinase by a restricted supply of GALA has also been reported for *F. oxysporum f.sp. lycopersici* and *F. oxysporum f.sp. cepae* (Cooper & Wood, 1975; Holtz & Knox-Davies, 1985a). In contrast, cellulase was not induced by monomeric glucose but by low concentrations of the glucose dimer, cellobiose. Similarly cellulase was induced in *T. reesei* batch cultures when CR was avoided by continuous feeding of cellobiose (Enari, 1983). Cellobiose is a logical 'choice' as the cellulase inducer *in vivo* in view of the universality of glucose in biological systems (Cooper, 1977).

A range of CWDE are produced by *V. albo-atrum* on extracted tomato cell walls (Cooper & Wood, 1975). The enzymes appeared in the order, *endo*-PG, *exo*-arabinase, *endo*-PL, *endo*-xylanase and cellulase (C_x). The sequential appearance of the enzymes may reflect the availability of inducers as the wall polymers as they were progressively degraded, beginning with the pectic matrix. This sequence would seem to be a general phenomenon as several other fungi including *F. oxysporum, C. lindemuthianum* and *B. allii* release CWDE in a similar order (Cooper & Wood, 1975; English, Jurale & Albersheim, 1971; Mankarios & Friend, 1980). *In vivo*, analogously, pectinases are the first enzymes to appear and cellulases the last as illustrated by several fungal-plant interactions, eg, oilseed rape cultivar Primor infected with *Leptosphaeria maculans*, *Verticillium* wilted lucerne and *Pyrenochaeta terrestris*-infected onions (Easton & Rosall, 1985; Heale & Gupta, 1972; Horton & Keen, 1966b). Enzyme production by entomopathogens grown on insect cuticle has recently been shown to follow a similar sequence, eg, *Metarhizium anisopliae, Beauvaria bassiana* and *Verticillium lecanii* release proteolytic enzymes substantially earlier than chitinolytic enzymes in cultures containing locust cuticle. The chitin microfibrils are less accessible to initial degradation because they are masked by protein, as shown by a chitin specific lectin which bound only to protein-extracted cuticles (St. Leger, Charnley & Cooper, 1986a). Once again, therefore, the first enzymes to be released corresponded to the major matrix polymer and subsequent production depended on the availability of inducers released during initial enzymolysis. CWDE are often synthesised at very low basal levels in absence of an inducer or under CR, although even *basal* synthesis may be lower in the presence of high levels of catabolites (Cooper & Wood, 1975). The action of basal levels of enzymes on large and sometimes insoluble wall polymers may

well be important for generating inducers for further synthesis of CWDE's. In this context *exo*-polysaccharases would be far more effective at releasing inducers than *endo*-enzymes (Cooper, 1977). Basal enzyme production, inducer release and sequential production may have important implications in tissue degradation and the mode of parasitism *in vivo* (see Literature Review 3.).

Synthesis of pectinases or cellulases may be constitutive and independent of the presence of an inducer, eg, *Aeromonas liquefaciens* and *Myrothecium verrucaria* (Hsu & Vaughn, 1969; Hulme & Stranks, 1971). Constitutive production is most commonly controlled by CR but there are examples of non-repressible, constitutively synthesised CWDE; the cellulase of *Pseudomonas solanacearum* and a PG and xylanase of *Helminthosporium maydis* (Bateman, 1976; Kelman & Cowling, 1965). The importance of these different levels of control in parasitism has not yet been realised (Cooper, 1986b) and will also be discussed in Literature Review 3.

Catabolite repression is non-specific and evoked by many readily metabolisable carbon sources, the repressing action of glucose ('glucose effect') is particularly well established. Repression of protease production may be caused by nitrogen and sulphur compounds as well as carbon sources (Cohen, 1973; R. St. Leger, pers. comm.).

The most significant genetic work on fungal CWDE production concerns the cellulase enzymes of wood rotting fungi, specifically *Trichoderma reesei* (Montenecourt et al., 1979) and *Polyporus adustus* (Eriksson & Goodell, 1974). Workers turned to these systems in hope of isolating strains with enhanced cellulase production for glucose generation and for fermentation into fuel alcohol (Brown, 1979; Eveleigh & Montenecourt, 1979; Montenecourt *et al.*, 1979; see Literature Review 2.4.). However, largely because of their simpler genetic conformation

and the ability to freely exchange markers with well characterised strains of *E. coli*, the greatest advances in pectinase regulation have occurred with the *Erwinia chrysanthemi* (Ech) and *E. carotovora* pectate lyase systems that are currently under investigation by a number of workers (Collmer et al., 1985; Keen, Dahlbeck, Staskawicz & Belser, 1984; Andro, Chambost, Kotoujansky, Cattaneo, Bertheau, Barras, Van Gijsegem & Coleno, 1984; Van Gijsegem, Toussaint & Schoonejans, 1985; Zink & Chatterjee, 1985).

In screening for cellulase-deficient mutants of *P. adustus*, several mutants were isolated that simultaneously lacked mannanase and xylanase (Eriksson & Goodell, 1974), which strongly indicated that this group of enzymes were regulated by one gene locus. It is possible that these strains were actually secretory mutants, which are the enzyme deficient mutants (E^-) most frequently isolated (Cooper, 1986a; see Literature Review 2.3. and Table 4). Simultaneous loss in activity was probably caused by pleitropic effects as the mutants of *P. adustus* secreted +Type levels of pectinase, laminarinase and β-glucosidase. Simultaneous induction of cellulase and mannanase has also been reported for *Chrysosporium lignorum* (Eriksson & Winell, 1968), and production of *endo-* and *exo*-cellulases of *Neurospora crassa* are also regulated by the same gene (Myers & Eberhart, 1966). Cross-induction of CWDE could reflect heterogeneity of defined substrates or non-specificity of enzyme assays, eg, *V. albo-atrum* PL and PG are moderately induced by rhamnose, which may contain trace levels of GALA residues (Cooper, 1977). Production of C_x, C_1 and β-glucosidase by a *T. reesei* mutant was concomitantly released from CR (Montenecourt *et al.*, 1979). Each enzyme of the cellulase complex was additionally under discrete control, to some degree; thus revertants were isolated from cellulase deficient mutants that produced β-glucosidase. Differentially enhanced levels of

β-glucosidase and C_x are produced by other mutants of *T. reesei* (Markkanen, Bailey & Enari, cited in Montenecourt *et al.*, 1979). Coordinate control of related enzymes may afford a greater adaptive efficiency to a fungus *in vivo*.

True pectinase isozymes are each coded by separate genes whereas their concordant transcription may be controlled at single loci. It is unlikely that the PG and PL activity of *V. dahliae* is determined by single enzymes considering the large number of isozymes produced by its close relative *V. albo-atrum* (Mohan & Ride, 1984). It is reasonable to postulate that PG and PL production may be under the control of at least 2 loci as PG^-PL^- mutants were obtained after repeated mutagenesis of mutants singly deficient in one type of pectinase (Howell, 1976). It remains to be seen, just how many structural genes code for the 2 PL and the numerous PG isozymes of *V. albo-atrum* (Cooper *et al.*, 1978; Mohan & Ride, 1984).

So far 5 genes coding for 5 of 12 possible isozymes, detected in cultures of *Erwinia chrysanthemi* (Ech), have been identified. However, it is thought unlikely that all 12 are the products of separate genes (see Literature Review 1.6.), (Collmer *et al.*, 1985; Kotoujansky *et al.*, 1985). Structural genes coding for 2 PGL isozymes have been cloned from Ech in *E. coli via* plasmids (Collmer *et al.*, 1985) and PL genes for isozymes b, c and d have been cloned out from a genomic library in λ phage (Kotoujansky *et al.*, 1985); results indicated the presence of 2 further genes for isozymes a and e. Interestingly there is strong evidence of homology between PGL isozymes b and c as shown by DNA-DNA hybridisation; these two neutral isozymes appear to be duplicates (Schoedel & Collmer, 1986). Furthermore, many strains of Ech express a pair of neutral isozymes, indicating strict conservation of the duplicates. It is therefore surprising that these enzymes are

apparently redundant in polygalacturonan utilisation and in pathogenesis (Roeder & Collmer, 1985; Schoedel & Collmer, 1986).

Molecular cloning of Ech gene libraries constructed in λ phage, has revealed that the PGL isozyme genes are arranged in two clusters (Kotoujansky, Diolez, Boccara, Bertheau, Andro & Coleno, 1985; Van Gijsegem *et al.*, 1985). Furthermore, Kotoujansky *et al.* (1985) found that the two structural genes for PME and PGL isozyme d were closely linked on the chromosome of Ech; these enzymes are probably transcribed together and could co-operate in tissue degradation. *Endo*-PGL and *exo*-PG are coordinately regulated in Ech which reflects their joint participation in polymer degradation and inducer generation (Collmer & Bateman, 1982), (see Literature Review 2.2., below).

2.2. Biochemical basis of pectinase regulation.

β-galactosidase synthesis by the *lac* operon of E. coli is mediated by an allosteric tetrameric protein which is the product of a regulator gene. This *repressor* binds to a receptor site called the *operator* which has the effect of shutting off enzyme synthesis. An inducer molecule binds to the repressor which causes the protein to undergo an allosteric change which releases it from the operator (de Cromburgghe, Chen, Anderson, Nissley, Gottesman & Pastan, 1971). β-galactosidase production is under negative control but there are examples of positive control, eg, arabinose metabolism in *E. coli*; the repressor is bound to the chromosome until the inducer (arabinose) is present, at which point the protein molecule undergoes a conformational change and allows transcription to procede (Sheppard & Englesberg, 1967). The operator locus was identified by its ability to mutate, resulting in a failure to bind to the repressor protein enabling the mutant to produce β-

galactosidase constitutively (Jacob & Monod, 1961). Constitutive mutants also arise from mutations in the repressor gene.

RNA polymerase binds to a separate promoter region from where it begins the sequential transcription of the 3 *lac* structural genes as a single polycistronic mRNA molecule. Cyclic adenosine 3', 5' monophosphate (cAMP) and its receptor protein (CRP) bind to a second site in the promoter region. cAMP has a key rôle in mediating synthesis, and the structural genes are not expressed in its absence. Many results suggest that CR depends on a reduction in intracellular levels of cAMP and indeed, production of a number of bacterial enzymes can be derepressed by the addition of cAMP to cultures (Pastan & Perlman, 1970; Cooper, 1977).

Endo-PGL production by *E. chrysanthemi* is induced in the presence of galacturonan and insoluble extracted plant cell walls (Chatterjee, Buchanan, Behrens & Starr, 1979; Collmer & Bateman, 1982). Induction is probably mediated by the oligomeric products released by basal levels of extracellular *endo*-PGL and *exo*-PG, as transport of the galacturonan polymer (MW 10 000-20 000) into the cell is unlikely (Collmer & Bateman 1982; Collmer *et al.*, 1982a). PGL activity is dependent on the presence of Ca^{2+} ions, but PGL is still produced on galacturonan when these ions have been removed by chelation with EDTA; this is because induction of PGL is mediated by saturated digalacturonic acid $(GALA)_2$ which is released by *exo*-PG. EDTA inhibits induction of PGL on cell walls because *exo*-PG fails to release $(GALA)_2$ and basal PGL is inactive in the absence of Ca^{2+}. Basal PGL activity releases (amongst other UGALA oligomers) unsaturated di-galacturonic acid and larger oligomers which are cleaved to $(GALA)_2$ by extracellular *exo*-PG. $(UGALA)_3$ and $(UGALA)_4$, taken up by Ech are also cleaved to $(GALA)_2$ and $(UGALA)_2$ by an *intracellular exo*-PL. The combined efforts of the two extracellular enzymes release inducers, and serve to make the induction process auto-

catalytic (Collmer *et al.*, 1982b). Excessive amounts of inducers lead to self-catabolite repression of PGL synthesis. CR of PGL synthesis in Ech and *Erwinia Carotovora* can be relieved by the addition of cAMP to the media (Fergusson & Chatterjee, 1981; Tsuyumu, 1979).

It is notable that mono-GALA is a far inferior inducer of PGL synthesis than the two dimers (Collmer & Bateman, 1981). The commercial availability of mono-GALA has made it an obvious choice for induction studies (eg Cooper & Wood, 1975) but as with Ech and *E. carotovora* it may be of lesser importance as an inducer than the saturated and/or unsaturated dimers.

The efficacy of an inducer may well be determined by its rate of uptake and metabolism. Additionally the rate at which a potential inducer is utilised may well determine the conditions under which CR is relieved and induction takes place.

Proteins have been identified in the periplasmic spaces of various bacteria that have affinities for specific sugars and amino-acids (Lehninger, 1975). Specific active transport mechanisms for uronic acids may well be present in bacteria and in the cytoplasmic membranes of fungi, as different sugars are assimilated at varying rates (Jennings, 1974; Rattigan & Ayres, 1977b), eg *Rhynchosporium secalis* has a high affinity (K_m) for glucose and GALA but a low affinity for galactose. Galactose was metabolised slowly and accumulated in the mycelium which additionally reduced the rate of assimilation. Uptake of GALA by *R. secalis* was severely reduced by metabolic inhibitors (Ayres & Olutiola, 1973) indicating the presence of an active transport mechanism. In contrast GALA uptake by *S. fructigena* was largely a passive process and little affected by temperature or metabolic inhibitors (Rattigan & Ayres, 1977a and b). Assimilation of trimeric UGALA by Ech was slower than dimeric UGALA which indicates a degree of

transport specificity. Alternatively the trimer, because of its larger size, is less amenable to uptake, which is borne out by the fact that larger oligomers are also assimilated slowly. However, the kinetics and specificity of oligalacturonide transport are likely to be a limiting step in the induction and repression processes in bacteria and fungi, as exemplified by catabolite resistant (CR^-) mutants which are simultaneously hyper producers of pectinases under conditions which are normally conducive to CR. Resistance to CR may well be as a result of a failure to take up the oligalacturonides rather than because of alterations to the repressor locus (Collmer & Bateman, 1981). This important part of *endo*-pectinase regulation warrants further study.

There is evidence that the products of the extracellular enzymes are not directly responsible for induction, but little is known about the metabolism of uronic acids in fungi and no potential inducer of pectinase synthesis has been identified. It is possible that the inducer is a product of extracellular enzyme acivity, eg, mono-GALA or UGALA, or that the uronic acids are converted to keto-uronic acids as in bacteria, or directly oxidised to dicarboxylic acid (Olutiola & Ayres, 1973; Hollman, 1964) which could induce pectinase production directly.

As yet no attempts have been made to induce pectinase synthesis in fungi with intermediates of uronic acid metabolism. It is possible that pectinase synthesis is mediated by similar mechanisms to those found in Ech model by Collmer *et al.* (1982b), as described below. When $(UGALA)_2$ is incubated with dialysed cell free extracts of Ech, the products of the intracellular enzymes are effective inducers of PGL (Collmer & Bateman, 1981). $(UGALA)_2$ is digested by oligogalacturonidase (OGL) to 4-deoxy-L-*threo*-5-hexulose uronic acid (DTH) which is in turn isomerised to 3-deoxy-D-glycero-2, 5-hexodiulsonic acid (DGH) and reduced to

2-keto-3-deoxy-D-gluconic acid. The last metabolite in this enzymically mediated pathway is subsequently converted to yield pyruvate and glyceraldehyde-3-phosphate (Collmer *et al.*, 1982b; Kilgore & Starr, 1959). The importance of OGL was emphasised when mutants deficient in OGL were found to be uninducible for PGL (Collmer *et al.*, 1982b). Ech PGL is not inducible in *E. coli* clones, reflecting the absence of *exo*-PG and OGL (Collmer *et al.*, 1985; Keen, Dahlbeck, Staskawicz & Belser, 1984). The OGL^- mutant CU2 produced +Type levels of PL when supplimented with DGH, therefore, the products of OGL are key elements in PGL induction. They additionally serve as repressors of PGL synthesis. High rates of formation of DTH and DGH lead to a reduction in cAMP levels and consequently to self imposed CR (Collmer *et al.*, 1982b). Synthesis of the *endo*-PGL/exo-PG complex of *E. carotovora* is also mediated by DTH which is produced by the action of an intracellular OGL (Stack *et al.*, 1980). The rate of induction depends on the efficiency of inducer uptake and/or metabolism; saturated $(GALA)_2$ is metabolised less efficiently by OGL than unsaturated $(UGALA)_2$. CR is imposed faster by the more readily metabolised dimer. A partially OGL-deficient mutant that metabolised the unsaturated dimer was hyperproductive for PGL in conditions that impose CR on the +Type, and, when CR was relieved, PGL production was less than the +Type.

The OGL-deficient mutant of Ech that was uninducible for PGL *in vitro* remained pathogenic and succeeded in macerating potato tissue (Collmer & Whalen, unpublished, cited in Collmer *et al.*, 1982b). This suggested that induced synthesis of PL was unnecessary for pathogenesis and that basal synthesis was sufficient to cause tissue degradation see Literature Review 3.). As this mutant was unable to utilise the products of PGL degradation the result also suggested that the primary function of PGL is not to supply the bacterium with nutrients during pathogenesis (Collmer *et al.*, 1982b).

2.3. Secretion of pectinases.

After translation, extracellular pectinases leave the cytoplasm by a passive process or *via* an active secretory system. Protein secretion in Gram-positive and Gram-negative bacteria has recently been reviewed by Pugsley & Schwartz (1985) and in fungi by Chang & Trevithick (1974). In eukaryotes, polypeptides synthesised on the ribosomes pass from the membrane of the rough endoplasmic reticulum (ER), *via* the smooth ER (Golgi), into vesicles which subsequently fuse with the cytoplasmic membrane to release the enzymes (Lampen, 1974). Enzyme secretion can be a very complex process; 23 post-translational complementation groups have been identified that control the secretory pathway of acid phosphatase and invertase in cell membrane vesicles of *Saccharomyces cerevisiae* (Novick, Field & Schekman, 1980). Yeast secretory mutants have revealed the importance of post-translational events in releasing synthesised polypeptides from the rough ER, prior to glycosylation. Precursors of carboxypeptidase and invertase remained firmly bound to the rough ER until the yeast mutants were returned to the permissive temperature for secretion (Ferro-Novick *et al.*, 1983; Ferro-Novick, Hansen, Schauer & Schekman, 1984).

In prokaryotes, enzymes are synthesised on cell membrane-bound ribosomes from where they are released into the periplasmic spaces by direct transfer through the cell membrane. Some bacterial enzymes may not take on their tertiary structure until after emergence from the cytoplasm (Lampen, 1974).

The accumulation of PGL in the periplasm of Ech Out$^-$ mutants and pectolytic *E. coli* clones indicates that export is a two stage process beginning with excretion from the cytoplasm. The signal peptide sequences on the PGLc and PGLe isozymes were present and were successfully processed by the *E. coli* clones (Keen & Tamaki, 1986). This, in

association with the presence of 'mature' PGL isozymes, suggests that the first step in secretion involves the well characterised *Sec* mechanisms (Collmer & Keen, 1986; Pugsley & Schwartz, 1985).

The second stage of PGL secretion, which is a controlled process by Ech and not simply the result of autolysis, has been analysed by the use of Out^- mutants. Genetic mapping has shown that the Out^- genes of Ech occur in three loci (Andro *et al.*, 1984; Thurn & Chatterjee, 1985). The Out^- mutation did not affect enzyme synthesis but the release of PGL, PME and C_x were reduced. However, protease was released at normal levels which suggests the existence of a separate secretory mechanism for export from the periplasm (Andro *et al.*, 1984). PGL of Ech a true extracellular enzyme as +Type Out^+ strains export >98 % (Chatterjee *et al.*, 1979; Collmer *et al.*, 1985). *E. coli* lacks the necessary Out genes and clones are unable to export Ech PGL from the periplasm (Collmer & Keen, 1986). The secretion process in soft-rot Erwinina pathogenesis is being further explored as there may be an important link between secretion and pathogenicity (reviewed by Collmer & Keen, 1986; see Literature Review 3.).

Before extracellular fungal pectinases encounter host tissue after release from the cytoplasm they must traverse the fungal cell wall. The enzymes may become physically bound to the cell wall or become lodged amongst the chitin fibrils which could have important consequences in terms of pathogenic strategy and localised tissue degradation (see Literature Review 3.). Enzyme release occurs most easily at the more porous, hyphal tips, thus, the MW of the enzymes concerned may well determine their rate of diffusion through the wall, eg a heavy invertase (MW 210 000) maximally leaked into the culture filtrates of *Neurospora crassa* when the hyphal tip : surface area ratio was high (Chang & Trevithick, 1974). Therefore inhibition of growth by

phytoalexins could stifle the secretion of some larger CWDE (Byrde & Archer, 1977), unless of course they were released as a result of autolysis.

2.4. Selection and *in vitro* application of mutants with altered capacity to regulate or synthesise pectinases.

Different classes of mutant, altered in their ability to synthesise or regulate the production of CWDE may be obtained by careful attention to screening procedures and particularly to the design of the plate media employed (Eveleigh & Montenecourt, 1979; Montenecourt & Eveleigh, 1979; Cooper, 1986a). The astute use of well characterised CWDE mutants *in vivo* may provide some insight into the importance of cell wall degradation during pathogenesis; this particular application of induced mutants will be described in detail in Literature Review 3. Genetical approaches have led to an improved understanding of regulation processes, as outlined above, but some regulatory mutants also have industrial applications, eg, for commercial enzyme production and saccharification of cellulosic waste (Brown, 1979; Faith, Neubeck & Reese, 1971; Montenecourt *et al.*, 1979). Consideration will be given in this section to the selection, variety and use of some of the CWDE mutants which are listed in Table 4.

Methods for inducing mutations in bacteria and fungi with chemical agents and UV irradiation are well documented (Esser & Keunen, 1967; Goodenough, 1978; Stent & Callender, 1978). Suspensions of bacteria and fungal spores are generally incubated in buffer containing the mutagen (1-10 % w/v), or shallow spore suspensions are irradiated with UV in petri dishes, and spread onto selection media after suitable dilution. Although the main effect of UV is to cause point mutations, large chromosomal aberrations do occur. Pyrimidine residues, most commonly

thymine-thymine, form stable dimers after exposure to UV and mutations are thought to result from the induction of an error-prone repair system, as demonstrated in *E. coli* (Smith, 1977). UV mutagenesis has been used to obtain pectinase deficient mutants of *V. dahliae*, *F. oxysporum f. sp. lycopersici* and *M. fructigena* (Howell, 1976; Howell, 1975; Mann, 1962; McDonnell, 1958; Puhalla & Howell, 1975). Because UV irradiation tends to disrupt large chromosome portions, simultaneously neutralising many genes, the use of chemical mutagenic agents is usually preferred. The alkylating agents N-methyl-N'-nitro-N-nitrosoguanidine (NTG) and ethyl methanesulphonate (EMS) are most commonly employed. NTG which is possibly the most powerful mutagen so far discovered (Adelberg, Mandel & Chen, 1965), promotes point mutations at a high frequency, often close to sites of DNA replication. NTG either has a direct mutagenic effect, causing tautomeric shifts and mispairing of bases, or indirectly leads to the operation of an error prone repair system (Drake & Baltz, 1976). Mutations arise from EMS treatment, as guanine and adenine bases are alkylated and also from base-pairing errors (Rhaese & Boetker, 1973). Although EMS is a less powerful mutagen, it does promote the formation of single point lesions at a much higher frequency than NTG (Zimmermann, Schwater & Loer, 1966). It is thus a preferable agent for induction of 'pathogenicity' mutants where a direct correlation between the lack of a 'pathogenicity' factor can only truly be made with a mutant altered in a single locus. NTG has been succesfully used to obtain stable auxotrophs of *V. dahliae* and *B. allii*, 'pathogenicity' mutants of *B. fabae* and CWDE mutants of Ech and *M. fructigena* (Hastie & Heale, 1984; Howell, 1976; Hutson & Mansfield, 1980; Andro *et al.*, 1984; Threlfall, 1971; Typas & Heale, 1976b). Cutinase-deficient mutants have been obtained for *C. lindemuthianum* using UV, NTG, EMS and nitrous acid

treatment (Dickman & Patil, 1986). A combination of mutagenic treatments (in series) is sometimes used to increase mutant variety (Montenecourt & Eveleigh, 1979).

As stated in Literature Review 1., pectinase activity is frequently conferred by numerous isozymes which are the products of separate structural genes. Clearly loss of a single isozyme gene product will be masked on an agar/pectin plate assay (see below) by the presence of the remaining unaffected isozymes. Total activity could be lost however by removal of a control gene (Cooper, 1986a; Montenecourt & Eveleigh, 1979) or by inducing mutations in the secretory mechanisms. However, specific isozymes may play key rôles in pathogenicity which would not be revealed by consideration of total activity, therefore problems of detecting the presence of single isozymes need to be circumvented. Attempts are being made to reintroduce cloned PGL isozyme genes (Collmer *et al.*, 1985; Kotoujansky *et al.*, 1985) that contain a mutation, induced *in vitro*, back into +Type Ech by marker exchange. Marker exchange mutagenesis (approach reviewed by Botstein & Shortle, 1985) of the PGLb (Roeder & Collmer, 1985a) and PGLc (Roeder & Collmer, 1985b) genes was accomplished with cloned PGLb and PGLc genes that had been inactivated *in vitro* by insertion of a kanamycin resistance marker. The capacity for PGL synthesis was found in several recombinants of Ech by conjugational transfer of a plasmid (Chatterjee and Starr, 1977), however, the sites of the regulatory locus or structural genes were not determined in the recipient strains. As yet no attempt has been made to clone pectinase genes of fungal pathogens. This reflects the infancy of fungal gene cloning systems, and the increased complexity of the fungal genome. High frequency transforming vectors have now been developed for *Aspergillus nidulans* (Ballance & Turner, 1985; Yelton, Hammer & Timberlake, 1984) and it is hoped that

similar vectors can be used, in association with selectable genetic markers, to isolate and clone fungal pathogenicity or virulence genes (reviewed by Yoder, Weltring, Turgeon & Van Etten, 1986); pisatin demethylase genes have already been succesfully cloned out of *Nectria haematococca* (Van Etten 1986, in Yoder *et al.*, 1986).

Transposon mutagenesis provides a more subtle approach to neutralising genes than by using traditional mutagenic agents. Transposons insert at specific sites on the genome and can carry antibiotic markers to aid selection. They have recently been used to obtain PGL deficient mutants of Ech (Andro *et al.*, 1984; Thurn & Chatterjee, 1985).

Screening media can be designed to maximise isolation of specific mutants. Substrate polymers are incorporated into an agar/salts medium to allow induction and action of specific enzymes around developing fungal or bacterial colonies, following inoculation. Extracellular enzymes are detected around colonies as cleared haloes in the agar; the undegraded substrate may require visualisation by precipitation or staining (Andro *et al.*, 1984; Chatterjee & Starr, 1977; Hankin & Anagnostakis, 1975; Montenecourt & Eveleigh, 1977). Developing agents are usually toxic, therfore, colonies must be replica plated prior to visualisation. Techniques that do not necessitate replica plating are more desirable, e.g, transformants of Ech that produced PGL were detected by their ability to sink into semi-solid pectate agar (Collmer *et al.*, 1985; Keen *et al.*, 1984) and blotting techniques have been developed for testing the presence of cell-bound enzymes (Collmer & Bateman, 1981; Ried & Collmer, 1985). Intracellular enzymes can be detected after colony autolysis or by the addition of appropriate degradative enzymes such as lysozyme or through incubation with phage (Collmer *et al.*, 1985; Kotoujansky *et al.*, 1985; Paoni & Arroyo, 1984).

Selection of some CWDE mutants can be enhanced by enrichment techniques similar to those employed for maximising isolation of auxotrophs (Demain, 1971; Masurekar, Kahgan & Demain, 1972). OGL^- Ech mutants were isolated after mutagenised bacterial suspensions had been incubated with saturated digalacturonic acid with the antibiotics ampicillin and D-cycloserine. +Type OGL^+ strains that utilised $(GALA)_2$ were killed by the antibiotics; putative OGL^- survivors were recovered in glucose/ salts media and further tested for OGL deficiency (Collmer & Bateman, 1981). In contrast, a strain of *E. carotovora* showing *enhanced* production of PGL was isolated by a chemostat enrichment procedure (Almengor-Hecht & Bull, 1978).

T. reesei colonies have been screened for catabolite repression resistance (CR^-) as this characteristic is often associated with hyper productivity (Demain, 1972). CR^- mutants have been isolated on cellulose agar that contain glycerol as the repressing catabolite because glucose cannot be used as it can inhibit cellulase activity (Montenecourt, *et al.*, 1979; Hoffman & Wood, 1986). Constitutive and CR^- mutants have also been obtained by growing colonies on cellulose agar in the presence of an antimetabolite, in this case a toxic analogue of glucose (2-deoxyglucose) which kills +Type strains that metabolise it. The mutants which produce cellulases constitutively survive by utilising glucose released from degradation of the cellulose. Hyperproducers have also been obtained from revertants of cellulase deficient mutants as a result of mutations induced in the control genes. These can be easily screened on a simple inducing media as revertants will grow and cellulase activity is revealed by the presence of a halo of degradation in the substrate (Montenecourt & Eveleigh, 1977a and b).

By using a combination of mutagenic treatments and screening techniques, Montenecourt *et al.* (1979) have isolated a single rapidly growing partially CR^- mutant from 800 000 colonies, which highlights the rarity and/or inefficiency of selecting useful mutants. This mutant produces 5 x more cellulase than the +Type. Workers are currently investigating the possibility of obtaining hyper-producing secretory mutants by screening for strains resistant to membrane active antibiotics (Eveleigh & Montenecourt, 1979; Montenecourt, *et al.*, 1979). Mutants that produce enzymes that are resistant to end-product inhibition would simplify and increase the efficiency of batch fermentation culture (Montenecourt *et al.*, 1979).

Considering the considerable market for pectinases for use in industrial processes (£165 million per annum; reviewed by Fogarty & Kelly, 1983) it is surprising that there are few examples where hyper-producing/catabolite resistant fungal or bacterial strains have been isolated, to optimise production (Fogarty & Kelly, 1983; Fogarty & Ward, 1972). Pectinases are not produced commercially from bacteria although potentially useful high producing constitutive strains are known (Fogarty & Kelly, 1983; Ward & Fogarty, 1974).

By adapting the pectinase screening media it should be possible to produce a range of mutants as outlined in Table 4. CR^-, PG^-, PL^- mutants of the fungal pathogens used in this work could throw light on the mechanisms regulating enzyme synthesis and on the contribution of pectinases to pathogenicity (Cooper, 1986b).

Table 4. Possible range of regulatory mutants and screening criteria. *a*

Mutant category	Selection criteria	Rationale
Catabolite repression resistant CR^-	Synthesis in presence of readily metabolisable repressors. Glucose added to inducing media, eg, polygalacturonan/salts.	Mutation in repressor gene leads to synthesis of enzyme in presence of excess glucose or degradation products (self CR).
Constitive I^-	Maximum synthesis in absence of inducer Activity present on non-inducing, non-repressing media, eg, for pectinase, cellulose/salts and a pectin overlay.	Mutants may arise from failure to produce repressor or possess abnormal operator.
Constitutive, non-repressible I^-CR^-	As above but in the presence of repressors, eg, pectin overlay on glucose salts media.	
Enzyme deficient E^-	Absence of activity on inducing media.	Mutants may arise from mutations in a (i) Structural gene. (ii) Regulator gene. (iii) Secretory system. (iv) Structural or control gene related to enzymes which are involved in generating inducers.

a adapted from Cooper (1986a).

3. The rôle of *endo*-pectinases in pathogenesis.

The first part of this section is a description of the rôle that *endo*-pectinases may take in host-parasite interactions as construed from evidence which is both diverse and extensive but largely circumstantial. This will lead to an endorsement of the need for a genetic approach in evaluating the significance of pectinases in different diseases, as illustrated by examples from parasites with contrasting strategies. The final section is a description of the three host-parasite systems used in this work and their expedience for establishing the importance of PG and PL to the pathogen during infection by way of induced mutants.

3.1. Detection of *endo*-pectinases in diseased tissue.

Endo-pectinases have been associated with bacterial and fungal soft rots for many years (Brown, 1915; de Bary, 1886; Tribe, 1955). As pectinases are produced by many pathogens numerous attempts have been made to link pectic degradation with symptom causation (reviewed by Bateman & Basham, 1976; Collmer *et al.*, 1982b; Cooper 1983; 1984; 1986b; Wood, 1961).

The isolation of pectinases from infected tissue provides a good basis for establishing a rôle in pathogenesis and there are many successful examples cited in the literature (Hancock *et al.*, 1964a; Scott & Fielding, 1985). However in some cases workers have failed to detect pectinase activity and for several possible reasons:

1. The enzymes are not produced by the pathogen *in vivo*.
2. Labile enzymes are denatured during extraction.
3. The enzymes have been deactivated or immobilised by ionic binding or inhibitors.

It is often assumed that necrosis occurs through wall degradation but this is not always the case as for example in Fire blight caused by *E. amylovora*. The pathogen fails to produce CWDE in liquid culture or *in vivo* (Cooper & Goodman unpublished, in Cooper, 1986) and cell leakage may be, in contrast to soft-rot Erwinia species, induced by the release of a toxin (D. Youle, pers. comm.) and not by the action of PGL. There have also been several reports of non-pectolytic enzymes being involved in the maceration process but none have been fully substantiated (Bateman & Basham, 1976; Chesson, 1980; Collmer, 1982). Alternatively, pathogens that produce pectinases *in vitro* may fail to do so *in vivo* because of CR evoked by host sugars (see Literature Review 2 and below) or as a result of unknown control mechanisms (Cooper, 1977).
Increased enzyme activity may also derive from the host (Langcake, Bratt & Drysdale, 1973), it is therefore essential to characterise the pathogen's enzymes, eg, by IEF. Furthermore, prudent extraction methods and purification techniques (eg column IEF) may release enzymes from inhibitors present in the host tissue (Fielding, 1981; see Literature Review 1.7.).

3.2. Symptom formation and the appearance of *endo*-pectinases.

It is important to show whether an increase in pectinase activity precedes or occurs concurrently with symptom formation as a CWDE may be irrelevant to pathogenesis and may be produced during a period of saprotrophy. PL levels were found to increase simultaneously with the onset of wilting in lucerne infected with VAA (Heale & Gupta, 1972). PG levels increased in and around lesions on *V. faba* leaves infected with *B. fabae* (Balasubramani *et al.*, 1971) and in stem canker lesions of oil seed rape infected with *Leptosphaeria maculans* (Easton & Rossall, 1985). PL activity was also detected in susceptible onion bulbs at the

onset of rot symptoms caused by *Fusarium oxysporum f. sp. cepae* (Holtz & Knox-Davies, 1985b) and coincidentaly with lesion formation in *Phaseolus vulgaris* hypocotyls infected with *C. lindemuthianum* (Wijesundera *et al.*, 1984).

Endo-pectinases and other CWDE appear in a number of diseases as outlined in the previous sections although their function depends largely on when and where they are produced during pathogenesis (see Literature Review 3.4.).

A constitutively produced *endo*-PG isozyme is present in the conidia of *B. cinerea* (Verhoeff & Liem, 1978; Verhoeff, pers. comm.) which implies that it may be involved at the outset of pathogenesis. Activity has also been found in ungerminated conidia of *Geotrichum candidum* and *Colletotrichum orbiculare* (Barash, 1968; Porter 1969).

Conidial bound cutinases, which may enable the spore to adhere to the leaf surface (Kolattukudy & Köller, 1983), have been conclusively shown to be involved in cuticular penetration during the early stages of infection (see Literature Review 3.4). Non-specific esterase activity has been found in conidia and appressoria of several fungi, eg *B. allii* and *Venturia inaequalis* (Kritzman *et al.*, 1981; Nicholson, Kúc & Williams, 1972) True 'cutinases' which solubilise long chain fatty acid esters are known to be produced by several fungal pathogens, the best characterised of which are those of *Fusarium solani pisi* and *B. cinerea* (Purdy & Kolattukudy, 1975; Shishyama, Arakai & Akai, 1970b; reviewed by Aist, 1983; Kolattukudy, 1983; Kolattukudy & Köller, 1983). The suberin layer in the endodermis may also be a substantial barrier to penetration. Suberin shows many similar properties to cutin and a cutinase-like esterase is produced by *F. solani f. sp. pisi* when grown on potato suberin which strongly resembles its cutinase in properties and activity (Fernando, Zimmermann & Kolattukudy, 1984). It remains to

be seen whether esterases are also a necessity for endodermal penetration.

3.3. Microscopic and chemical evidence for wall-modification by pectinases *in vivo*.

A reduction in GALA content of infected tissue is also a good indicator of pectolytic activity (Cooper, 1983), eg, 82 % and 90 % of pectic substances were removed from sunflower stems by *Sclerotinia sclerotiorum* and from bean hypocotyls by *S. rolfsii* and *R. solani* respectively (Bateman, 1976). Free GALA has also been detected in the infection droplets of *B. fabae* on *Vicia faba* leaves (Balasubrami, Deverall & Murphy, 1971).

Dissolution of cell walls, membrane disruption and organelle disorder may occur well in advance of colonisation in tissue infected with necrotrophic pathogens. Rapid wall swelling and degradation occurs around infection pegs of *Botrytis cinerea, B. fabae* and *Helminthosporium maydis* and in spreading lesions caused by *B. allii* and *B. squamosa* in onion epidermis (Mansfield & Hutson, 1980; Mansfield & Richardson, 1981; McKeen, 1974; Stewart & Mansfield, 1985; Wheeler, 1977). Such an effect would be expected if the interlinking rhamnogalacturonan chains had been dissolved by the action of *endo*-pectinases, but, although these pathogens produce high levels of these enzymes in liquid culture, the evidence for involvement in infection by these and many other pathogens yet to be established.

3.4. *Endo*-pectinases and infection strategy.

Extracellular *endo*-pectinases are highly destructive against plant tissue, as described in Literature Review 1.4. and below. However, wall degradation and tissue maceration by *endo*-PG may be limited when the

enzyme is hyphal-bound as demonstrated by immunogold labelling of *C. lindemuthianum* PG. The enzyme remained bound to the hyphal surface in infected bean tissue (O'Connell, Bailey, Vose & Lamb, 1986) and no enzyme activity was detectable, following conventional extraction. This restricted activity coincides with the biotrophic phase of infection. Similarly, localised degradation by obligate biotrophs is also achieved by tightly bound CWDE's as evident from ultrastructural studies of infection pegs from appressoria of *Bremia lactucae* and appressoria of *Erysiphe graminis* (Ingram, Sargent & Tommerup, 1976; Sargent & Gay, 1977). The subtle growth of biotrophs through primary cell walls probably depends on glycanases and glycosidases that are largely hyphal-bound and detectable at only very low levels. It is notable that the macerating *endo*-pectinases are not produced by biotrophs (Cooper, 1986b; D. Longman, pers. comm.).

As stated in Literature Review 2.1. there may be a link between the major matrix polymer and the major CWDE's that a pathogen uses in degradation. This is well exemplified by the obligate biotrophs *Ustilago maydis* which causes maize smut and by the bean rust pathogen, *Uromyces fabae*. The bean pathogen produces mainly arabanases and galactanases which coincide with key matrix polymers of its dicot host but the main CWDE's of *U. maydis* are β-1,4-xylanase, β-xylosidase and α-arabinosidase which compliment the principal matrix polymer of cereal walls (Cooper, 1983; Cooper, Harding & Wilson, unpublished cited in Cooper, 1984; Cooper, 1986b; D. Longman, pers. comm.). Other facultative cereal parasites produce a similar array of CWDE (Cooper, 1986b). Enhanced levels of xylanase and α-arabinosidase were found in maize infected with *U. maydis*; the enzymes probably originated from the pathogen as they had similar isoelectric points as those produced *in vitro*. The degradation products of hyphal-bound enzymes would be

released directly into the path of the extending hyphae, establishing a readily available carbon source; in addition to providing a means for traversing the host wall, enzyme binding would appear to be a very economical application of CWDE.

Many facultative biotrophs that produce high levels of CWDE *in vitro* do not inflict noticeable damage to the host during the initial stages of colonisation. Most pathogens assume a mixed strategy of parasitism and are hemibiotrophic (Cooke & Whipps, 1980), eg, *L. maculans*, *Septoria* species, *F. oxysporum f. sp. cepi*, *Rhynchosporium secalis* and *C. lindemuthianum* (Easton & Rossall, 1985; Hilu & Bever, 1957; Holtz & Knox-Davies, 1985a & 1986a; Jones & Ayres, 1974; Wijesundera *et al.*, 1984; Durrands, unpublished results). Hemibiotrophy is usually associated with leaf parasites (Cooke & Whipps, 1980) but the root invading vascular parasite VAA could also be regarded as hemibiotrophic, because root invasion is not accompanied by cell killing and symptoms do not appear for *c* 10-20 days after inoculation (Bishop & Cooper, 1983; Cooper, 1981; Cooper, 1986b). The hemibiotrophs characteristically colonise their hosts in a conservative manner without causing apparent damage. A destructive necrotrophic phase then ensues after the initial period of biotrophy. This change can be very sudden and may be due to many causes but it often coincides with the appearance of pectic enzymes, wall degradation and suppression of host defences, eg, phytoalexin production (Cooke & Whipps, 1980; Cooper, 1986b).

Cooper (1977; 1983 & 1986b) has postulated that *endo*-PL production by *C. lindemuthianum* begins as catabolite repression is relieved when the available free sugars have been consumed. This would seem tenable as necrosis often coincides with sporulation which creates a greatly increased demand for nutrients (Cooke & Whipps, 1980). Pathogenesis, PG and cellulase levels of *Pyrenochaeta terrestris* was closely related to

onion sugar concentration (Horton & Keen, 1966b), however, a tomato cultivar did not resist attack by *P. lycopersici* although sugar levels in the roots remained high enough to repress CWDE production *in vitro* for 4 d.

Evidence for the repression of PL synthesis in Fusarium infected onion bulbs by free sugar has been provided by the recent work of Holtz & Knox-Davies (1985c and 1986b). Apoplast sugar concentration in equivalent parts of isogenic susceptible and resistant cultivars of onion plants were very similar, however bulb scales, leaf sheaths and stem plates of the same plants showed marked differences in sugar concentration. Resistance to infection in the leaf sheaths and bulb scales was positively correlated with high levels of glucose, fructose and sucrose but replenishment of the sugars from the symplast occurred more slowly in the stem plate region, which was, coincidentally, the susceptible area succesfully colonised by *Fusarium oxysporum f. sp. cepae*. High sugar levels, equivalent to those found *in vivo*, repressed PL production *in vitro* (Holtz & Knox-Davies, 1985c and 1986a).

Further results suggested that the change in sugar levels during infection by this facultative biotroph has a profound effect on the change from biotrophy to necrotrophy (Holtz & Knox-Davies, 1986b).

The question arises as to why the biotrophic mode does not continue and why continued successful colonisation of the host is only facilitated by necrosis. As the fungus has an adequate supply of carbon for growth, restriction probably involves the presence of a host produced inhibitor (Holtz & Knox-Davies, 1986b). Extensive colonisation depends, as it does for successful infection by many necrotrophs (See below), on the ability of the fungus to kill host cells by the production of pectic enzymes. In a sense the apoplast sugars could be viewed as preformed inhibitors of fungal development.

Furthermore, changes in pH and ionic conditions in infected tissues may enhance the activity of *endo*-pectinases (Cooper, 1986b). The pH of onion sap infected with *F. oxysporum f. sp. cepi* rose from 4.4 to 7.5 during pathogenesis (Holtz & Knox-Davies, 1985a). Such an alkaline shift, which was coincident with a rise in *endo*-PL activity and a corresponding decline in *exo*-PG activity, has been noted in tissue infected with other Fusarium species (Bateman, 1966; Hancock, 1968; Mullen & Bateman, 1971). Onion stem tissue that had a low pH was also more resistant to infection by Fusarium (Holtz & Knox-Davies, 1985a). Alternatively in lesions of *S. sclerotiorum* on sunflower the pH fell from 6.2-4.5 which favoured the production and activity of PG (Hancock, 1966; Bateman & Basham, 1976). pH reduction was probably facilitated by the release of oxalic acid which additionally chelated Ca^{2+} ions, rendering the polygalacturonan more amenable to the PG.

As a parasite tips the balance of its association with a host it may be exposed to phytoalexins or other fungitoxic agents. By killing the host tissue in the early stages of infection through the production of *endo*-pectinases a pathogen may prevent the accumulation of these compounds. This pre-empting of host defences is central to the strategy of necrotrophic parasites.

The use of *endo*-pectinases as a means of offence is well demonstrated by many necotrophic bacteria and fungi. Clearly *endo*-pectinases will, in most cases, also have a rôle in the nutrition of necrotrophs as potential metabolites are generated as a consequence of tissue degradation.

Necrotrophic parasitism is well illustrated by members of the genus *Botrytis*. *B. fabae* penetrates the cuticle of *V. faba* leaves 5-6 h after inoculation and grows rapidly through cell walls which swell in advance of fungal colonisation (see above). Host cell death occurs

around penetration points and as the forming lesion spreads. Incompatible species, eg, *B cinerea, B. tulipae* succeed in penetrating the leaves but kill far fewer cells in the non-host *V. faba*. Differences between *B. fabae* and species avirulent to *V. faba*, have been attributed to the greater tolerance of the bean pathogen to wyerone derivatives and its superior ability to detoxify the inhibitors (Rossall & Mansfield, 1978). Virulence, as determined by the ability to cause spreading lesions, was strongly correlated with an ability to kill host tissue (Hutson & Mansfield, 1980). The significance of rapid and extensive cell killing may lie in suppression of phytoalexin synthesis (Cooper, 1986b; Mansfield, 1980). 52 NTG induced avirulent mutants of *B. fabae* that were equally insensitive to wyerone acid were incapable of causing extensive damage to the host. Unfortunately no attempt was made to determine why these strains were less able to kill the host cells or, to identify the loss in the mutants of a pathogen-produced toxic factor. *Endo*-pectinases are produced by all of these *Botrytis spp. in vitro* and *in vivo* (Balasubramani, *et al.*, 1971; Hancock *et al.*, 1964a and b) and are likely to be the primary cause of cell death.

Endo-pectinases of necrotrophs tend to be synthesised rapidly at the outset of infection. Basal levels are sometimes so high that induced production may not be necessary for successful parasitism (Andro *et al.*, 1984; Collmer *et al.*, 1982b; Cooper, 1986b).

Many saprotrophic bacteria eg *Pseudomonas fluorescens* produce *endo*-pectinases but may remain non-pathogenic. Rapid production of *endo*-pectinases relative to the rate of wound healing or phytoalexin production is characteristic of the pathogenic pectolytic bacteria (Andro *et al.*, 1984; Collmer *et al.*, 1982b; Lapwood, 1957; Wood, 1967; Zucker & Hankin, 1970). However, differentiation between saprotrophic and necrotrophic fungi is probably much more complex and determined by

the outcome of several stages of recognition and host-parasite challenges (Heath, 1985).

As stated previously, high levels of pectic enzymes may lead to tissue destruction and suppress resistance. In contrast, pectic enzymes, at non-toxic levels, may elicit phytoalexin production. Pectic oligomers released by *E. carotovora* PGL and *Rhizopus stolonifer* PG induced the release of phytoalexins either directly, as endogenous elicitors, or as a result of cell damage (Davis, Lyon, Darvill & Albersheim, 1984; Nothnagel, McNeil, Albersheim and Dell, 1983).

As synthesis of a parasite's pectic enzymes may be repressed *in vivo* by the presence of free sugars in host tissue, an advantage would be conferred to a necrotroph by having non-repressible constitutively produced or high non-repressible basally produced enzymes. However, although few have been examined, organisms that show constitutively produced CWDE occur at a very low frequency (Bateman & Basham, 1976; Kelman & Cowling, 1965; Cooper, 1983). The importance of constitutive production in pathogenesis is unknown.

The significance of *endo*-pectinases in disease remains largely unresolved although they have been under scrutiny for many years, whereas results from studies on *F. solani f. sp. pisi.* cutinase have provided an almost complete picture of the rôle that this enzyme takes during infection of *Pisum sativum*. The cutinase work illustrates the need for an all round approach to be undertaken with a host-parasite interaction involving pectinases.

Studies were initiated from ultrastructural evidence which suggested that cuticle hydrolysis was involved in penetration (Aist, 1976; Dodman, 1979; Verhoeff, 1980). As the main physical barrier to penetration is the polyester, cutin, a search began for a relevant degradative fungal enzyme (Shishyama *et al.*, 1970a and b); cutinase

activity has been detected in the culture fluids of many different fungal pathogens, eg, *B. cinerea, B. squamosa, Colletotrichum graminicola, Fusarium* species, *Pythium ultimum, R. solani* and *S. rolfsii* (Baker & Bateman, 1978).

Briefly, the cutinase of *F. solani f. sp. pisi* is not produced constitutively but is induced on extracted cuticles and by low levels of cutin monomers (Lin & Kolattukudy, 1978). It has a pH optima of 9-10 and releases oligomers from the substrate polyester. These oligomers are further broken down by the same enzyme, which appears capable of multiple attack (Kolattukudy & Köller, 1983). All fungal cutinases so far isolated are of a similar size (MW ≃ 25 000).

Direct evidence of involvement in penetration has been achieved by the use of specific enzyme inhibitors and by labelling with ferritin coated anti-bodies. Treatment of inoculation sites with ferritin-labelled anticutinase demonstrated that the fungus secreted cutinase during penetration (Shaykh, Soliday & Kolattukudy, 1977). Inhibitors such as diisopropyl fluorophosphate prevented infection and penetration, as did rabbit antisera prepared against the purified cutinase (Köller, Allan & Kolattukudy, 1982; Maiti & Kolattukudy, 1979). Infection of papaya fruit by *C. gloeosporioides* was similarly prevented (Dickman, Patil & Kolattukudy, 1983).

When sensitive radio-immune assays were used to determine cutinase in spores of virulent and avirulent strains of *F. solani* high cutinase levels were correlated with virulence (Köller *et al.*, 1982). Most significantly the rôle of cutinase has been confirmed by the failure of two cutinase deficient mutants, isolated after mutagenic treatment, to penetrate or infect intact papaya fruit (Dickman & Patil, 1986). The latter approach will be discussed in the following two sections.

3.5. Use of specific mutants to determine the rôle of pathogenicity factors in disease.

There are few examples of successful attempts to identify determinants of pathogenicity by using mutagenesis, which probably reflects a lack of detailed knowledge of either the disease interactions or the genetic background of the pathogens. The alternative approach is to induce and isolate strains that are *non-pathogenic* and then establish what factors have been lost (Daniels, Barber, Turner, Cleary & Sawcyz, 1984; Hutson & Mansfield, 1980). This latter approach relies on a rapid and reproducible pathogenicity screen. Non-pathogenic isolates of *Xanthonas campestris* were selected after NTG treatment by their inability to cause symptoms on turnip seedlings. Work is in progress to clone the genes that are responsible for conferring pathogenicity to the +Type strains (Daniels, Barber, Turner, Sawczyc, Byrde & Fielding, 1984).

However, the importance of tomatine in resistance of tomato fruits to infection by *F. solani* has been resolved by the former approach, by isolating tomatine-tolerant mutants through mutagenising and plating out spores of an avirulent tomatine sensitive strain onto media containing tomatine (Défago & Kern, 1983). Genetic analysis has confirmed that tomatine tolerance is carried by a single gene (Défago, Kern & Sedlar, 1983).

The significance of pisatin demethylation and tolerance for succesful parasitism of pea by *Nectria haematococca* has been established by sexual mating of virulent and avirulent strains followed by analysis of the progeny (Van Etten, 1981). Genetic analysis also showed that virulence is determined by many genes, although pisatin tolerance and demethylation are coded by 2 separate loci.

3.6. The use of specific mutants to determine the rôle of *endo*-pectinases in disease.

The latest genetic transformation techniques are now being applied to bacterial pectinase producing pathogens (see Literature Review 2.4.) and this should resolve the importance of pectolytic degradation, at least in the soft-rots. Gene transfer techniques have recently been established for *Aspergillus* and it is encouraging that these may one day be used to isolate pectinase genes of fungi. The removal of pathogenicity determinants of fungi rather than from bacteria would seem a more worthwhile pursuit considering the complex nature of fungal diseases of plants in comparison with the relatively clear cut bacterial soft-rot systems.

Many attempts have been made to correlate pectinase production *in vitro* with pathogen virulence or pathogenicity (Keen & Erwin, 1971; Mohan & Ride, 1984; Morrall, Duczek & Sheard, 1972); Talboys & Busch, 1970). The relevance of such results must be put into question as little attention was paid to the pH and the substrates used in the media and the genetic variation that exists between naturally occuring isolates. A direct correlation between PG, PL and PG/PL production and pathogenicity or virulence can only be established using of mutants of a well characterised +Type that are deficient in these enzymes (Cooper, 1983). Ech mutants deficient in PGLb or PGLc retained their pathogenicity which suggests a redundancy for these two isozymes (Collmer & Keen, 1986; Roeder & Collmer, 1985a and b; Schoedel & Collmer, 1986). In contrast, the rôle of PGL in maceration by Ech was positively confirmed by the conjugational transfer of a plasmid coding for the lyase gene (Chatterjee & Starr, 1977). A degree of synergism between pectinases in maceration is suggested by Roberts, Berman, Allen, Stromberg, Lacy & Mount (1986a and b). *E. coli* strain L-757 carried two insertion

plasmids pDR1 and pDR30 containing, respectively, two *E. carotovora* *endo*-PGL genes and an *endo*-PGL plus an *exo*-pectate lyase gene; the gene products were able to macerate potato slices. As the enzymes were released into the tissue, the relevant Out genes may also have been inserted into the *E. coli* clone which suggests clustering between the PGL structural genes and the secretory (Out) genes.

In contrast the gene products could have been released by autolysis over the 48 h incubation period of the *E. coli* in the potato slices. A rôle for these enzymes in *pathogenesis* could be affirmed by reinsertion of these plasmids after *in vitro* mutagenesis.

As stated in section 3.1 extensive secretion by a pectolytic bacteria may be the key characteristic of pathogenicity. Out$^-$ mutants of a virulent +Type Ech strain, which accumulated PGL and C_{∞} in their periplasmic spaces, were isolated after mutagenic treatment (Andro *et al.*, 1984). These mutants failed to infect the test plant, *Saintpaulia ionantha* (Andro *et al.*, 1984).

Most attempts at correlating induced pectinase deficiency in fungi and pathogenicity has involved the vascular wilt pathogens *V. dahliae* and *F. oxysporum f. sp. lycopersici*. McDonnell (1958) and Mann (1962) isolated mutants of *Fusarium*, after UV irradiation, that grew poorly on agar containing pectin. The mutants, which were shown to lack PG, retained their pathogenicity, it was thus concluded that this enzyme was unimportant for successful infection. However, no attempt was made to determine the rôle of the potentially important PL, also produced by *F. oxysporum* (Cooper & Wood, 1975; Jones, Anderson & Albersheim, 1972). Puhalla & Howell (1975) using a pectate plate selection medium (Hankin & Anagnostakis, 1975) induced PG deficient mutants of *V. dahliae* which also retained pathogenicity. The selection method for PG deficency used by Puhalla & Howell was an improvement on previous work, as PG

production was detected directly in zones of degradation in the substrate rather than as a retardation of colony growth which could result from unrelated mutations. However, the mutants were only deficient for the part of the overall PG activity, which was optimal at pH 6.0. After repeated mutagenesis mutants were obtained which were almost completely deficient for PG at pH optima peaks of 6.0 and 4.0 (Howell, 1976). Although these different pH optima suggest the presence of at least two PG isozymes, unfortunately no further attempt was made at characterisation. PL and PME-deficient mutants were isolated; and mutants simultaneously deficient in PG and PL were also obtained. All of these mutants retained their pathgogenicity for cotton. The presence of a polygalacturonate lyase was simultaneously lost to mutants that were deficient in PL. Rather than discovering the production of a PGL by *V. dahliae* it is likely that the workers were actually assaying pectin lyase activity on polypectate. Pectin lyases from other fungi eg, *V. albo-atrum* (Cooper *et al.*, 1978), have been shown to degrade unmethylated polygalacturonan.

Unfortunately no attempt was made to determine the effect of pectinase deficiency on root invasion as the plants were inoculated by stem injection. It may be notable that although the symptoms shown by the plants infected with +Type and mutants were equally severe, wilting was delayed in plants infected with the various pectinase-deficient mutants (Howell, 1976). This suggests that although PG and/or PL may not be determinants of pathogenicity they may be involved in virulence.

Suprisingly, virulence of *S. fructigena* was significantly correlated with α-L-arabinofuranosidase production rather than PG as determined by CWDE-deficient mutants induced by Howell (1975). However, the statistically analysed results do suggest that the two enzyme activities are mutually beneficial in pathogenesis.

3.7. Choice of pathogens.

Three fungal pathogens were chosen which had contrasting parasitic strategies; *B. allii*, a necrotroph; *C. lindemuthianum*, a hemibiotroph and *V. albo-atrum*, a vascular parasite. Each produces *endo*-pectinases but the function of the enzymes may be different in each case and it should be possible to select mutants deficient in PG or PL after mutagenesis. The enzymes may be *pathogenicity* factors or *virulence* factors. In the former case a mutant lacking pectinases would be incapable of infecting the host, in the latter loss in pectolytic ability may result in lessening of the severity of symptoms and reduced colonisation.

3.7.1. *B. allii*.

B. allii is one of the causal agents of the economically important neck rot disease of onion (*Allium cepa*), (Maude, 1980). It is an aggressive necrotroph which forms spreading lesions on onion bulb tissue from inoculation sites (Stewart & Mansfield, 1985a).

PG has been implicated as the cell killing factor involved in colonisation and tissue destruction in necrotic diseases caused by *Botrytis* species but the evidence remains largely circumstantial (Kritzman *et al.*, 1981; Stewart, 1983; see Literature Review 3.2.).

B. allii is known to produce PG in liquid culture and in onion leaf tissue (Hancock *et al.*, 1964; Mankarios & Friend, 1980). No time course of production was attempted *in vivo* and the control of PG synthesis was not established by these workers. Furthermore, although PL activity was detected by Mankarios & Friend (1980) no attempt was made to characterise or to determine the rôle of this enzyme(s) in disease.

As *B. allii* produces almost entirely uninucleate spores (Threlfall, 1971) it is the most amenable species of *Botrytis* for obtaining stable mutants after mutagenesis, because the chance of complementation and/or

heterokaryosis should be greatly reduced. In addition the translucent single cell layers of epidermal cells which are so easily removable from onion bulb tissue are ideally suited for microscopic studies of disease development and cell killing.

PG and/or PL deficient mutants would be suitable for establishing a direct correlation between the production of the enzymes and their rôle in cell killing.

3.7.2. *C. lindemuthianum*

C. lindemuthianum is a hemibiotrophic parasite which causes large water soaked lesions on susceptible cultivars of *Phaseolus vulgaris*. Infection hyphae exhibit a period of biotrophy followed by maceration of host tissue which is accompanied by the production of PL (Bailey, 1982; Wijesundera *et al.*, 1984).

PL production may be repressed by the presence of host sugars during the biotrophic phase of infection (see Literature Review 2.1. and 2.4.). CR^- mutants would presumably release PL during initial colonisation and symptoms would form without the biotrophic phase. PL^- mutants could be used to determine the significance of PL in necrosis. *C. lindemuthianum* also has uninucleate spores and may also be amenable to genetic analysis by crossing as a sexual stage has now been demonstrated (Batista & Chaves, 1982). Thus, combinations of pectinase genes could be transfered into progeny and linkage groups could be identified.

3.7.3. *V. albo-atrum*

V. albo-atrum produces a range of specifically induced CWDE of which PG and PL are capable of killing tomato and potato tissue *in vitro* and causing wilt symptoms in tomato cuttings (Cooper *et al.*, 1978; Cooper &

Wood, 1980; see Literature Review 2.1.). The enzymes are induced by low concentrations of monomeric GALA, polygalacturonan and host cell walls; production is repressed in the presence of excess GALA and glucose (Cooper & Wood, 1975).

The position of PG and PL in the highly complex wilt syndrome remains controversial (Pegg, 1981; Puhalla & Bell, 1981). The recovery of pectinases from diseased tissue has proved to be difficult but PL has been found in lucerne prior to the onset of symptoms caused by *V. albo-atrum* (Heale & Gupta, 1972) and in tomato stem segments 3-4 d after infiltration with spores (Cooper & Wood, 1980).

Endo-pectinases may be of particular importance because host xylem vessels are characteristically occluded by pectic gels which originate from exposed middle lamella at pit membranes and perforation plates (Beckman, 1969; Beckman & Talboys, 1981; Cooper & Wood, 1980; Dimond, 1970; Van der Molen, Beckman & Rodehorst, 1977). Gel formation often precedes wilting or reduced vascular flow (Beckman & Halmos, 1962; Heale & Gupta, 1972) and may be responsible for causing the characteristic water stress. Other symptoms may arise indirectly from degradation of host walls (Cooper, 1984). Leaf abscission and epinasty may result from the generation of C_2H_4, and chlorosis from the production of H_2O_2 (Mussell & Strand, 1977) following the release of glucose oxidase. Solubilised peroxidase may be responsible for the extensive vascular browning and may additionally contribute to chlorosis and wilting by the indirect release of C_2H_4 (Cooper & Wood, 1980; Street & Cooper, cited in Cooper, 1986).

For the reasons given in the previous section there is still a need for fresh evidence to confirm the importance of pectinases in vascular wilt diseases by direct correlation (Cooper, 1983) which can be provided by PG^- and PL^- mutants of a pathogenic +Type strain of *V. albo-atrum*. *V.*

albo-atrum is also suitable for mutagenesis as it has predominantley uninucleate spores (Hastie & Heale, 1984) and established techniques for parasexual analysis (Hastie, 1964 and 1968; Hastie & Heale, 1985; Typas & Heale, 1978) may be employed for studying linkage between the various pectinase genes.

The following sections describe the attempts made at inducing, selecting and characterising PG and PL-deficient mutants of *B. allii* and *V. albo-atrum*, unfortunately time did not allow for extensive work on *C. lindemuthianum*. The regulation and properties of *B. allii* and *V. albo-atrum* PG and PL were also thoroughly examined. The characteristics and production of these enzymes is discussed in relation to the studies on PG and PL-deficient mutants of *V. albo-atrum* and pathogenicity.

Materials and Methods

1. Culture of Fungi.

1.1. Isolation and Maintenance.

The Wild Type *Verticillium albo-atrum* (VAA) was provided by Prof. Pegg of Reading University. Isolates of *Botrytis allii* were the gift of Dr. Maude of The National Vegetable Research Station at Wellesbourne. *Verticillium dahliae* isolates were provided by Dr. Clarkson of Bath University.

VAA was maintained on Czapek-Dox agar at 23°C and *B. allii* was grown under black-light conditions (16h photoperiod) on Yeast Extract Peptone (YEP) medium and medium X (MX) (Last & Hamley, 1956) at 18°C. Stocks of VAA and *B. allii* were maintained at 4°C on Corn Meal Agar and YEP slopes respectively. All Wild Type and mutant strains were also maintained under liquid nitrogen at -181°C.

1.2. Growth Media.

The inorganic salts solution described by Cooper & Wood (1975) was the basis of liquid media for production of inoculum and studies of enzyme production *in vitro*. It contained litre^{-1}, $NaNO_3$, KH_2PO_4, $MgSO_4$; (ppm), $FeSO_4.7H_2O$ 0.2, $ZnSO4.7H_2O$ 1.0, $NaMoO_4.\ 2H_2O$ 0.02, $CuSO_4.5H_2O$ 0.02, $MnCl_2.4H_2O$ 0.02.

Cultures were grown at *c* pH 5.0 or 8.0 with the non-metabolisable buffers 2-(*N*-morpholino)ethanesulphonic acid (MES) and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES) (0.05-0.1 M) respectively, unless otherwise stated.

1.3. Shake cultures.

1.3.1. Unrestricted cultures.

Shake cultures were used to study production of cell wall degrading enzymes (CWDE) *in vitro*. 100-200 ml basal salts solution containing a carbon source was dispensed into 350 ml Erlenmeyer flasks. After inoculation the flasks were shaken in a rotary incubator (150 rpm) at 25°C for up to 14 d.

1.3.2. Restricted cultures.

Carbon sources were either supplied in an "unrestricted" manner at 0.2-3 % (w/v) or the supply was restricted to the approximate rate of utilisation by the fungi, by means of diffusion capsules (Pirt, 1971). These continuous feed devices consist of cylindrical nylon containers that can be completely filled with a concentrated solution and sealed at one end with a semi-permeable membrane. When placed in a liquid medium and agitated the solute is released at an almost constant rate until the internal concentration is reduced by *c* 65 %. Capsules containing glucose or saturated galacturonic acid (GALA) were immersed in shake cultures. The rate of release of thes sugars was controlled by altering solute concentration and/or the number of membranes (from Visking tubing 8/32") at the open ends of the capsule. The combined effect of these factors on diffusion rates is shown in Fig 1.

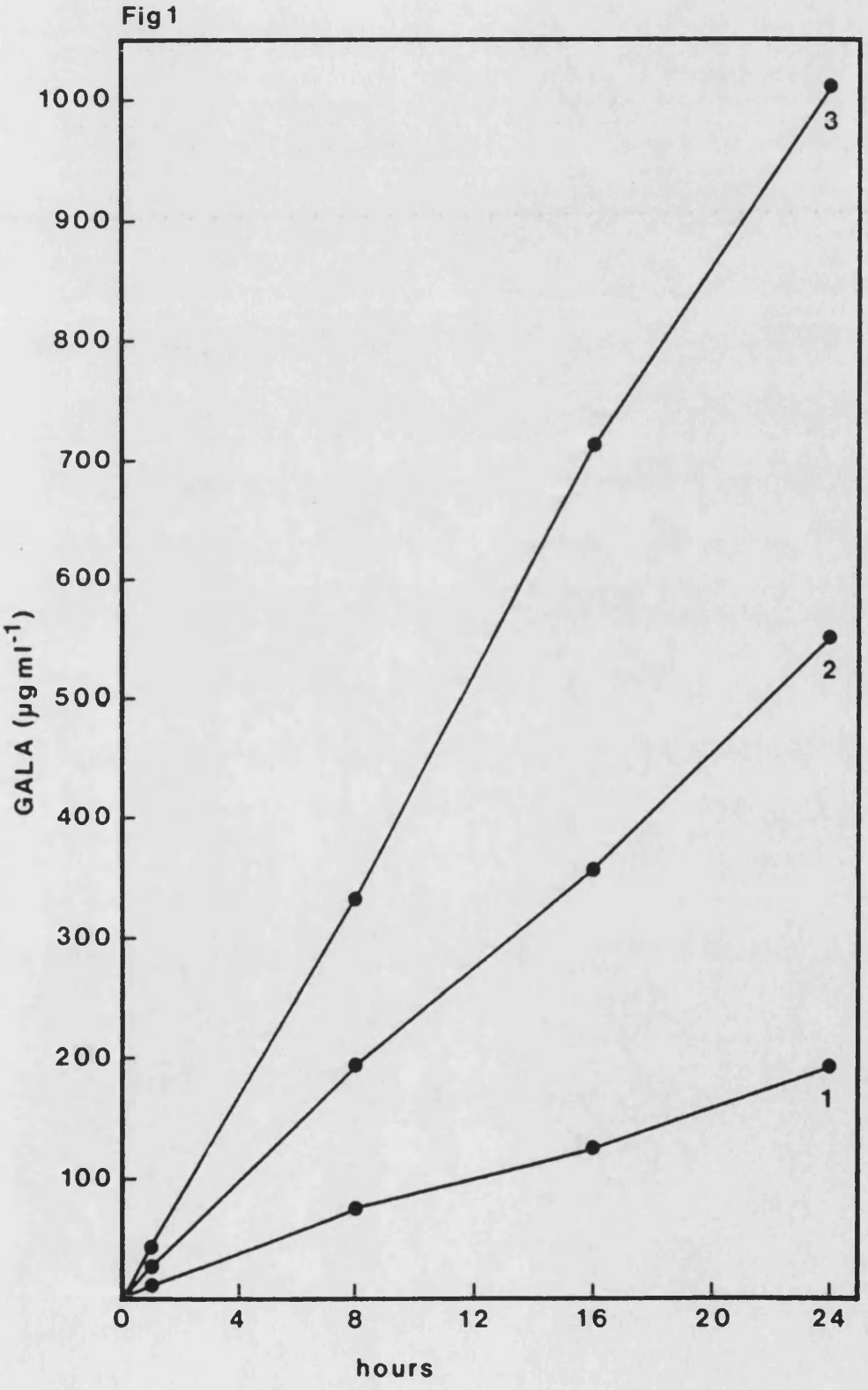
1.4. Inoculation of shake cultures.

1.4.1. Unrestricted cultures.

Spore suspensions or 5 mm agar plugs were prepared from 7-14 d. Czapek-Dox (VAA) or YEP plates (*B. allii*). Unless otherwise stated the

Fig 1. Release of galacturonic acid from diffusion capsules.

Release of galacturonic acid from diffusion capsules into 100 ml culture volume, shaken at 150 rpm at 25°C.
(1) 7.5 % (w/v) initial GALA concentration, 1 membrane layer; (2) 15 % GALA, 1 membrane; (3) 30 % GALA, 1 membrane.
Membrane orifice diameter 4 mm. These combinations released GALA at 8.0, 23.0 and 43.3 $\mu g\ ml^{-1}\ h^{-1}$. Release of glucose from capsules followed a very similar pattern.



inoculum per flask was 1 x 10^7, as determined by an improved Neubauer haemocytometer, or 1 agar plug.

1.4.2. Restricted cultures of *B. allii*.

Shake cultures containing 100 ml of glucose 1 % (w/v) basal medium, inoculated as above, were incubated for 48 h when extensive growth as small (*c* 1 mm) pellets was evident. Mycelium removed from six individual flasks by centrifugation (1 800 x g, 15 min) was used as the inoculum for each restricted culture. The total weight of mycelium from each flask was *c* 25 mg.

1.5. Measurement of fungal growth.

Aliquots were taken from flasks, filtered through dried pre-weighed Whatman # 1 filter paper, dried to a constant weight at 70°C and cooled in a desiccator prior to weighing.

B. allii grows exclusively as pellets and growth was measured in terms of mg dry weight $flask^{-1}$ at culture harvest. VAA grows as mycelial pellets, conidia and as blastospores. Growth was usually measured as final mg dry weight $flask^{-1}$; on insoluble tomato cell walls growth of strains was determined by blastospore production as estimated on a Neubauer haemocytometer.

1.6. Culture filtrate analysis.

Aliquots from each flask were filtered as described above and after centrifugation (1800 x g, 15 min, 4°C) the filtrate was analysed for galacturonic acid (GALA) and unsaturated GALA (UGALA) (Ayres, Papavizas & Diem, 1966); glucose, when employed as a carbon source was determined by the glucose oxidase assay (Fleming & Pegler, 1963).

Protein in solution was estimated by ultraviolet absorption (Kalb & Bernlohr, 1977) and by the following equation:

Protein concentration ($\mu g\ ml^{-1}$) = 183 A_{230} - 75.8 A_{260},

where A_{230} and A_{260} are the absorbances at 230 and 260 nm.

Filtrates were assayed for enzyme activity after dialysis at 4°C for 24 h in Visking tubing 8 or 12/32" diam. against c 200 vol. stirred distilled water at pH 6.5.

2. Sterilisation.

2.1. Culture media.

Except where indicated, all culture media, buffers, salts solutions, were sterilised by autoclaving at 121°C for 15 min. Media containing carbon sources were autoclaved at 115°C for 10 min. Media >pH 7 were sterilised in two components, the carbon source was added to the buffered alkaline salts solution after cooling; this limited caramelisation of sugars or breakdown of pectic polymers.

2.2. Proteins.

Proteins were sterilised by membrane filtration (Oxoid membrane filters) under reduced pressure.

2.3. Diffusion capsules.

Diffusion capsules were washed in absolute ethanol prior to addition of solutions, then autoclaved at 115°C for 10 min.

3. Measurement of pH.

pH readings were taken on a Pye Unicam Model 290 MK 2 meter.

4. Buffers.

The following were used in extraction and assay of enzymes and prepared

as described by Dawson, Elliot, Elliot & Jones (1969).

Britton Robinson Universal buffer: pH 2.6-12.0.

Citric acid-sodium citrate (0.1 M): pH 3.0-6.2.

N-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES) (0.05-0.1 M): pH 6.8-8.2.

2-(*N*-Morpholino)ethanesulphonic acid (MES) (0.1 M): pH 5.2-7.2.

K_2HPO_4-KH_2PO_4 (0.1 M): pH 5.8-8.0.

Tris-(hydroxymethyl)aminomethane-HCL (0.05 M): pH 7.2-9.1.

The non-metabolisable buffers MES and HEPES were used to maintain shake flasks at c pH 5.0 and 8.0 for promotion of PG and PL production respectively.

5. Enzyme substrates and inducers of synthesis.

5.1. Host cell walls.

5.1.1. Onion.

Cell walls were prepared from whole-sale Dutch onions, cultivar Rijhnsberger, by the method of Mankarios & Friend (1980) although the anti-foaming agent *n* octanol was substituted for suspended silica.

5.1.2. Tomato.

Cell walls of the VAA susceptible cultivar Craigella GCR 26 were prepared by the method of Laborda, Archer, Fielding & Byrde (1974).

5.2. Polygalacturonides.

Sigma sodium polypectate (NAPP) and pectin were used as the inducers of PG and PL in both shake cultures and in solid selection media. NAPP (Sunkist) was used for viscometric assay of PG as it is relatively more

viscous and less prone to precipitation than the Sigma products. Sigma pectin was used for viscometric, thiobarbituric acid (TBA) assay and UV absorbance assay for PL activity.

5.3. Carboxymethylcellulose.

CMC was supplied by BDH.

5.4. P-nitrophenol and naphthyl derivatives.

PNP derivatives of -α-L-fucopyranoside, -β-D-galactopyranoside, -β-D-glucopyranoside, -β-D-mannopyranoside, -β-D-xylanopyranoside were supplied by Sigma. α and β naphthyl phosphate and L-leucine-β-naphthylamide HCL were also obtained from Sigma.

6. Enzyme assays.

pH optima for *B. allii* PG and PL were determined and used in assays. All assays were performed in duplicate.

For VAA enzymes the pH optima reported by Cooper, Rankin & Wood (1978) were used in assays.

6.1. Polygalacturonase.

PG was assayed viscometrically in Technico 200 viscometers at 25°C (Cooper & Wood, 1975). Activity is given as Relative Viscometric Units (RVU) defined as 1000 x the reciprocal of time (min) for a 50 % decrease in relative viscosity of 8 ml of 1 % NAPP solution, buffered at pH 5.0 (0.1 M citrate) and 2 ml enzyme solution.

6.2. Pectin lyase.

PL was assayed by the thiobarbituric acid (TBA) procedure as adapted by Ayres *et al.* (1966). Reaction mixtures contained 2.5 ml 0.5 % buffered

pectin (HEPES or Tris 0.05 M pH 8.0 for *B. allii* PL and Tris 0.05 M pH 9.0 for VAA PL), 1.5 ml enzyme solution and 0.5 ml 0.09 M $CaCl_2$ (excluded for assaying *B. allii* PL). After an incubation period of 1 h 4 ml 0.04 M TBA : 1 N HCl (2 : 1) was pipetted into the reaction mixture which was then boiled for exactly 30 min to develop colour, measured at 550 nm. The two reference controls contained (1) enzyme solution and water and (2) substrate and water. Activity is expressed as $\mu g\ ml^{-1}\ h^{-1}$ UGALA released ml^{-1} enzyme in reaction mixture.

B. allii PL was also assayed viscometrically at 25°C with 1 % pectin (HEPES 0.05 M, pH 8.0) and by the direct measurement of the formation of the unsaturated uronide product (Albersheim & Killias, 1962).

0.9 ml of 0.1 % pectin buffered at pH 8.0 (HEPES 0.05 M) was pipetted into a quartz cuvette containing 0.1 ml of enzyme solution. Activity was determined by measuring the increase in optical density at 238 nm in a Shimadzu spectrophotometer. By using the molar extinction coefficient of the unsaturated bonds produced (4600) (Nagel & Anderson 1965) the OD can be expressed as μmol UGALA $ml^{-1}\ min^{-1}$.

6.3. Cellulase.

Cellulase activity (Cx) was assayed viscometrically with 0.21 % CMC in citrate buffer (pH 5.0, 0.1 M) (Cooper & Wood, 1975) and is expressed as RVU.

6.4. Glycosidases.

α-L-fucopyranosidase, β-D-galactopyranosidase, β-D-glucopyranosidase, β-D-mannopyranosidase, β-D-xylanopyranosidase were determined by measuring the rate of hydrolysis of *p*-nitrophenyl glycosides (PNP) (Jones & Bateman, 1972). 0.9 ml of PNP-glycoside in citrate buffer (0.2 $mg\ ml^{-1}$, 0.1 M buffer, pH 5.0) was dispensed into 0.1 ml enzyme and

incubated at 30°C for 1 h. 0.5 ml NH_4OH was then added to halt the reaction and develop the colour which was measured at 400 nm.

6.5. Leucine arylamidase.

Leucine arylamidase activity was determined by a modification of the method of Nakadai, Nasuno & Iguchi (1973), (R. St. Leger, pers. comm). 0.1 ml enzyme and 1 ml of a 1 mM solution of L-leucine-β-naphthylamide in Britton and Robinson Universal buffer (pH 7.0) was incubated for 1 h at 30°C. The reaction was stopped by the addition of 1 ml of 0.7 % (v/v) HCl in ethanol and 0.06 % (w/v) Fast blue BB). The OD was read at 540 nm after the mixture had stood for 10 min. Activity is expressed as OD of reaction mixture h^{-1} incubation as a measure of β-naphthylamide.

6.6. Peroxide formation.

Peroxide formation was detected around *B. allii*, colonies grown on a haemoglobin agar medium, prepared by the method of Koenigs (1974). Peroxide production was inferred when a green discolouration developed beyond or under colonies (Koenigs, 1974).

6.7. Enzymatic characterisation using the API-ZYM system.

The enzymatic profiles of *B. allii*, mutant and +Type VAA strains were determined using the semi-quantitative API-ZYM system (Slots, 1981). The 19 enzyme substrates and activities are listed in Appendix 5. 50 µl of filtrate from 7 d shake flasks were pipetted to each of the 20 cupules of the API-ZYM gallery which was enclosed in a moist chamber at 30°C for 4 h. After incubation, 25 µl of API reagents A and B was added to each cupule. The colour reaction was read after 5 min. The reagents used to detect enzyme activity were:

Reagent ZYM A:

Tris(hydroxymethyl-)aminomethane	25 g
Hydrochloric acid	11 ml
Lauryl sulphate	10 g
Distilled water	to make 100 ml

Reagent ZYM B;

Fast blue BB	0.35 g
2-Methoxyethanol	to make 100 ml

Arbitrary units 0-5 were assigned as guided by the colour chart provided by the manufacturers (API); where 0 is deemed to be a negative reaction and 5 to be maximal. At least two replicate API strips were employed for each isolate tested.

7. Paper chromatography.

The mobility of UGALA residues relative to GALA from *B. allii* PL reaction mixtures were analysed by descending paper chromatography (Partridge, 1949) on Whatman # 1 paper eluted with ethyl acetate : acetic acid : water : formic acid, 18 : 3 : 4 : 1 (Collmer & Bateman, 1982) for 20 h. 1 ml samples were removed periodically from 0.1 % pectin (pH 8.0, HEPES 0.05 M) and PL, incubated at 25°C and boiled for 5 min. After lyophilisation long chain pectic polymers were precipitated out with 200 µl 80 % ethanol to prevent tailing. *C* 100 µl of each sample was applied to the origin line at 5 cm intervals in a laminar flow cabinet. Spots on chromatogram strips were detected with silver nitrate (Trevelyan, Procter & Harrison, 1950), although 10 % sodium thiosulphate was used in place of ammonia as the fixative (D. Longman, pers. comm.).

8. Thin Layer chromatography.

To determine the size of the GALA residues released from NAPP by *B. allii* PG, 1 ml samples were periodically removed from 0.1 % NAPP (pH 5.0, MES 0.05 M) reacting with PG at 25°C and boiled. The samples were

lyophilised and resuspended in 200 µl of water. It was found to be unnecessary to clean the sample with ethanol (see above); in addition the ethanol had the undesirable effect of removing oligomers larger than the tetramer. 50 µl samples were applied to Merck silica TLC plates (2 mm thickness) in a laminar flow cabinet with micro-caps. Plates were first washed and then eluted for 4 h with the solvent system 'C' employed by Cooper *et al.* (1978) which consists of butanol : formic acid, 2 : 3. GALA (0.4 %) was used as a marker and the spots were detected by spraying with either aniline diphenylamine phosphate (Swinburne & Corden, 1969) or TBA spray (Warren, 1960); the latter is more specific for uronides (Cooper, Rankin & Wood, 1975). The aniline diphenylamine phosphate spray was very sensitive for detecting GALA oligomers released by PG. TLC plates were photographed and analysed on a Joyce-Loebl densitometer (Chromoscan 3).

In contrast analysis of degradation products of pectin by PL proved more difficult. Only faint blue or red spots were apparent on heating (100°C, 10 min, due to the insensitivity of both the staining reagents towards UGALA.

9. Enzyme purification.

9.1. Fractional precipitation with ammonium sulphate.

Ammonium sulphate was used to salt out proteins from the extracellular fluid of fungal cultures grown on pectin, CMC, glucose, and cell walls. The method was modified from Cooper (1974). Solid $(NH_4)_2SO_4$ was added to stirred filtrates to a final concentration of 95 % and stirred overnight at 4°C. Precipitated protein was collected from the filtrate, by centrifugation (23 000 x g 20 min, 8°C). Precipitates were dissolved in a minimum quantity of water (pH 6.0 dialysed overnight at 4°C

against 200 vol distilled water and made upto equal volumes before enzyme assay or storage at -20°C.

9.2. Isoelectric focusing.

9.2.1. Column focusing.

9.2.1.1. Broad range (pH 3-10).

Protein samples obtained from the ammonium sulphate precipitation step were exhaustively dialysed before fractionation by isoelectric focusing (IEF) in a 110 ml column (LKB 8101). The pH gradient (pH 3-10) was provided by carrier ampholytes at a final concentration of 1 % (w/w). The apparatus was set up as described in the manufacturers instructions and operated at 4°C. The following solutions were used:

1) Cathode solution: 1 pellet (c 0.1 g) of NaOH in 10 ml d. H_2O.

2). Anode solution: 0.2 ml conc. H_3PO_4, 15 g sucrose and 12 ml d. H_2O.

3) Light gradient solution: 1.3 ml Pharmacia "Pharmalyte" ampholine pH 3-10 in 51.7 ml sample and d. H_2O.

4) Dense gradient solution; 27 g sucrose, 1.4 ml ampholine and 35.7 ml d. H_2O.

The sucrose gradient was formed and introduced into the column as described by the manufacturers. VAA and *B. allii* produce acidic pigments in pectin culture and so the cathode was placed at the top of the column (Cooper & Wood, 1975) ensuring that any precipitation did not disrupt the density gradient in the rest of the column.

The power pack (LKB 2103) was set initially at 0.4 KV, 3 W. The voltage was increased to 1 KV after the current had fallen to c 5 mA. The run was continued until the current stabilised at c 3 mA. After a total run

time of c 40 h the column was emptied with a peristaltic pump and 4 ml fractions were collected at 4°C on an LKB redirac. The pH of the fractions was determined immediately at 4°C. Carrier ampholines and sucrose were removed by dialysis (24 h, 4°C) in 0.1 M KCl (200 vol) and then in distilled water (200 vol) to remove KCl (24 h, 4°C). Samples were made up to equal volume and assayed for enzyme activity or stored at -20°C.

9.2.1.2. Narrow range (pH 8-10.5).

Dialysed PL fractions (pH 7.5-11.0) obtained by broad range IEF were pooled and further fractionated by narrow range IEF (pH 8.0-10.5) according to manufacturer's instructions. The following solutions were used:

1) Cathode solution: 15 g sorbitol, 5.5 ml 1 M NaOH, 9.5 ml d. H_2O.

2) Anode solution: 10 ml 0.01 M CH_3COOH.

3) Light gradient solution: 0.3, 0.1, 0.3 ml Pharmacia "Pharmalyte" ampholines pH 9-11, 8-10, 6-8 respectively.

4) Dense gradient solution; 27 g sorbitol, 2.1 ml Pharmacia "Pharmalyte" ampholine and 34.9 ml d. H_2O.

Narrow range IEF was performed according to the manufacturer's instructions in an 110 ml LKB 8100-1 column. The anode was placed at the top of the column. Fractions were collected and dialysed as reported above for broad range IEF.

9.2.2. Flat bed isoelectric focusing.

Isoelectric focusing of isozymes was performed on an LKB2117 multiphor in ready-prepared LKB thin-layer (1 mm) polyacrylamide gels (PAG plates) which contain carrier ampholytes to give pH range 3.5 to 9.5. PAG plates and the multiphor were handled according to manufacturer's instructions. 19 μl samples containing 1-5 mg protein ml^{-1} were

applied to the gel adjacent to the cathode with small segments (*c* 10 mm^2) of chromatography paper. After 1 h the voltage was gradually increased from 0.25 KV to 1.50 KV and maintained at this for a further 30 min. The sample application papers were removed after 30 min to prevent tailing of the bands. The pH gradient was determined at points along the gel with a pH surface electrode. The zones were refocussed for 10 min after measuring the pH.

9.2.2.1. Polygalacturonase activity staining on flat bed PAGE plates.
The method was adapted from those of Lisker & Retig (1974) and Mohan & Ride (1984). Following IEF, gels were placed in 0.1 M citrate buffer (pH 5.0). After 5 min the buffer was replaced with 1.2 % NAPP (Sigma) in 0.1 M citrate buffer (pH 5.0) and incubated for 30 min at 30°C. The NAPP solution was discarded and the gels gently washed with two changes of distilled water. 1 % cetyltrimethyl ammoniumbromide (CTAB) or 0.05 % ruthenium red were used to precipitate and stain areas of undegraded NAPP. Contrast between the red stained background and the cleared zones indicating direct PG isozyme activity was improved on incubation overnight at 30°C. Gels suspended in water have been preserved for more than 12 months. Although the red stain fades, the NAPP can be restained at a later date if desired. The positions of clearing zones were carefully noted and the pI's determined. Gels were also scanned using a Joyce-Loebl densitometer (Chromoscan 3).

9.3. Sephadex G-100 gel column chromatography.
Enzyme samples from IEF were further purified by Sephadex G-100 descending chromatography using a 60 x 2.6 cm column (LKB) at 4°C, set up as described in the manufacturer's instructions. The top adapter was connected *via* a three way valve to a Mariotte flask which served as the

buffer reservoir. The bottom adapter was connected to an LKB Redirac *via* an LKB peristaltic pump.

The general procedure for perfoming gel filtration was as that described by Pharmacia in their manual "Gel Filtration, Theory and Practice". The Sephadex G-100 superfine was supplied as a dry powder. Before use it was swollen in an excess of K phosphate buffer (0.1 M, pH 6.5) for 7 h at 95°C. The surface layer of the gel, once settled and cooled to 4°C, was decanted so that the remaining suspension was a thick slurry.

The gel bed was poured in one continuous operation and stabilised by allowing 2-3 column volumes of degassed K phosphate buffer to be passed down the column. The flow rate was controlled by a peristaltic pump at 6 ml h^{-1}. The settled gel had a bed height of 47 cm giving a volume of 249.5 cm^3. 2 ml of bue dextran 2000 (1 mg ml^{-1}) was applied to the top of the gel *via* the three way adapter and eluted at 5 ml h^{-1}. The dextran moved through the gel in a narrow band indicating that the gel bed was uniform. In addition the large polymer allowed determination of void volume (V_o = 87 ml).

2 ml enzyme samples were applied to the gel and 4.6 ml fractions were collected. Fractions were assayed for enzyme activity and protein (absorbance at 230/260 nm) after dialysis. 0.005 % Merthiolate (w/v) was added to prevent microbial growth.

10. Determination of molecular weights and Stokes' radius (R_s).

10.1. Molecular weights.

The molecular weight of enzymes was estimated by gel filtration with Sephadex G-100 (Andrews, 1964) and the following marker proteins: ribonuclease (MW 13 700, Stokes' radius R_s 16.4 Å), chymotrypsin A (MW

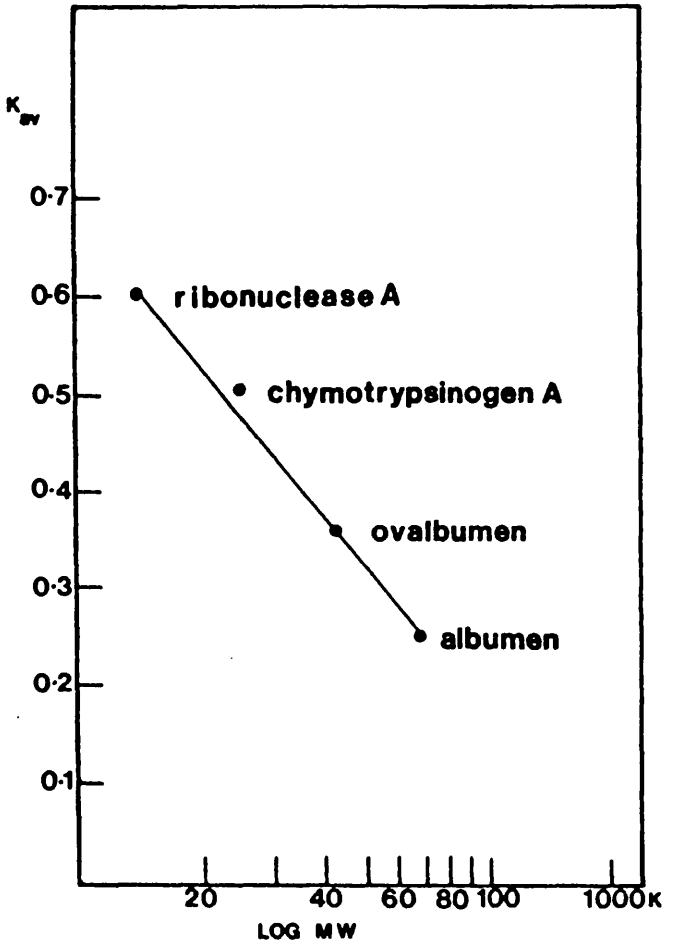


Fig 2. Calibration curve using molecular weight markers on a Sephadex G-100 column.

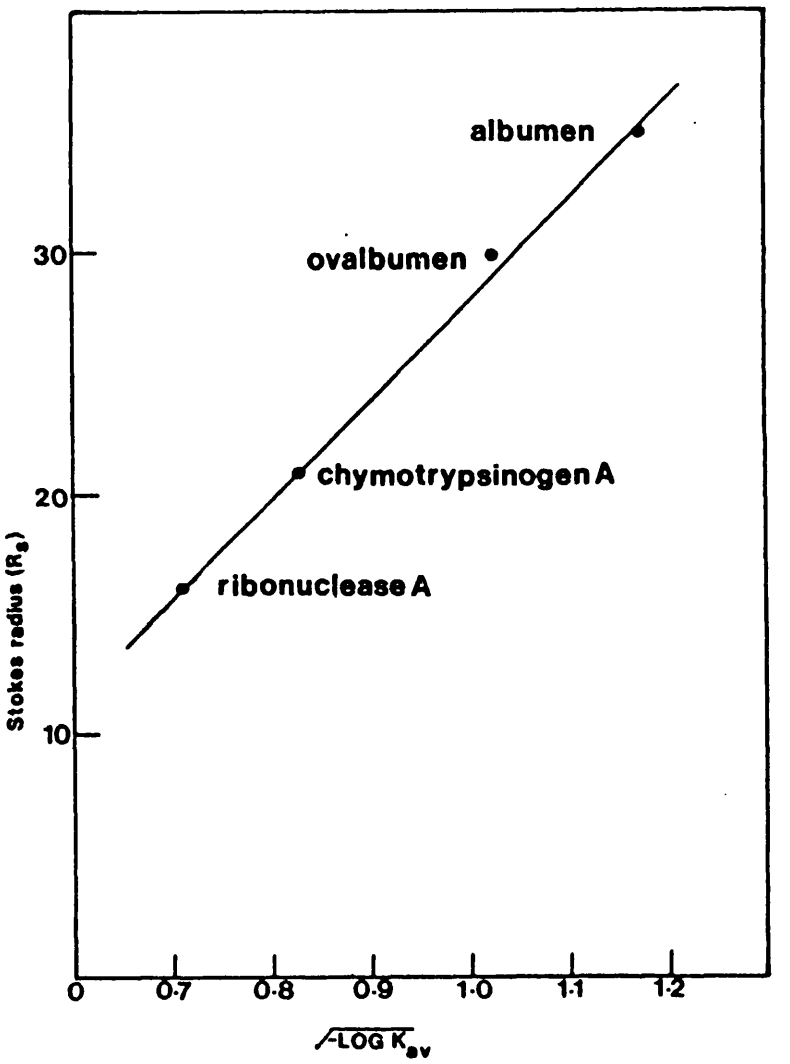


Fig 3. Calibration curve using R_s markers on a Sephadex G-100 column.

25 000 R_s 20.9 Å), ovalbumin (MW 43 000 R_s 35.5 Å) and aldolase (MW 158 000 R_s 48.1 Å). The standards were dissolved in 0.1 M K phosphate buffer, pH 6.5 and loaded two at a time on a column of Sephadex G-100 (2.6 x 47 cm) equilibrated with the same buffer. A flow rate of 5 ml^{-1} h^{-1} was used. For each standard and enzyme the diffusion coefficient $K_{av} = V_e - V_o/V_t - V_o$ was calculated (Laurent & Killander, 1964), where V_e is the protein elution volume and V_o and V_t the void and total volumes, respectively, of the gel bed (see previous section), For the standards K_{av} was plotted against MW on semi log. paper (Fig 2) and from the curve the MW of each enzyme was calculated.

The standard proteins formed a straight line over the molecular weight range 13 700 to 67 000 daltons. The useful range of the column could be extended upto 100 000 daltons. Results provided are from one column but almost identical results were obtained from a second, similar column.

10.2. Stokes' radius (R_s).

Stokes' radius (R_s), "the radius of a sphere of equal area to the enzyme", was calculated by gel filtration using the Sephadex column and marker proteins listed above. For each standard and the enzymes -log (K_{av}) was calculated, where K_{av} is the diffusion coefficient. For the standards the resulting values were plotted against R_s (Fig 3). From the curve the R_s of each enzyme was calculated.

11. Extraction of enzymes from infected onion tissue.

Host tissue, both infected and healthy, was extracted with 0.2 M KCl in Na acetate buffer (pH 4.5, 0.1 M) at the rate of 10 ml g^{-1} of tissue. The neutral detergent, Brij 35 was added (0.005 % w/v) to aid the release of the pectinases from the host tissue (Lisker, Katan, Chet & Henis, 1975). After blending at high speed for 5 min in a Waring

blender the extractant was first filtered through Whatman # 1 filter paper and clarified by centrifugation (23 000 x g, 4°C) for 15 min. The supernatant was dialysed overnight in 200 vol distilled water and concentrated by ammonium sulphate precipitation as described above. Enzymes were fractionated by broad range IEF Materials and Methods 9.2.1.1.

12. Mutagenesis.

12.1. *V. albo-atrum.*

In an attempt to promote the formation of mutants damaged by only single point lesions the alkylating agent ethyl methane sulphonate (EMS) was used in a procedure adapted from that used by Al-Aidroos & Seifert (1980) for the entomopathogenic Hyphomycete, *Metarhizium anisopliae*. Survival curves (Fig 4) for VAA conidia exposed to 1 % EMS were established in the following way. Spores from 10 d old Czapek Dox plates in sterile K phosphate buffer (pH 7.0, 0.05 M) were filtered through Whatman #1 filter paper to remove hyphae, and diluted in the same buffer to a concentration of 1 x 10^7 ml^{-1}. 1 ml of the spore suspension was added to 9 ml buffer containing 100 µl EMS (Sigma). Treatments were carried out at 22 and 28°C in shaking water baths. 1 ml samples were periodically removed and dispensed into buffer containing 10 % (w/v) sodium thiosulphate to quench the activity of the EMS (C. Soper, pers. comm.), rendering the ester to the non-mutagenic and less toxic sulphonic acid. After an incubation period of 45 min the spore suspension was diluted to 1 x 10^2 ml^{-1}. 1 ml aliquots were dispersed on 14 cm petri dishes of Czapek Dox media which were incubated at 23°C until colonies appeared after 3 d. Colonies from surviving conidia were scored on the third and fourth day after mutagen treatment because

Fig 4. Survival rate of EMS treated +Type *V. albo-atrum* conidia.

Conidia were incubated with EMS (1 % w/v) at 22 (◆) and 28°C (●) for up to 600 min. Samples of *c* 100 mutagenised conidia were plated onto Czapek-Dox media and incubated at 23°C.
Each point represents the mean number of colonies scored on 10 replicate plates after 4 d, expressed as a percentage of the mean number of colonies scored on the control plates (*c* 100 colonies). Control plates contained untreated conidia which were *c* 100 % viable.
Mean results of 2 replicate experiments.

Fig 5. Survival rate of NTG treated +Type *B. allii* conidia.

Conidia were incubated with NTG (100 μg ml^{-1}) at 24°C for up to 30 min. Samples of *c* 100 mutagenised conidia were plated onto YEP media and incubated at 18°C.
Each point (●) represents the mean number of colonies scored on 10 replicate plates after 3 d, expressed as a percentage of the mean number of colonies scored on the control plates (*c* 100 colonies). Control plates contained untreated conidia which were *c* 100 % viable.
Mean results of 2 replicate experiments.

Fig 4

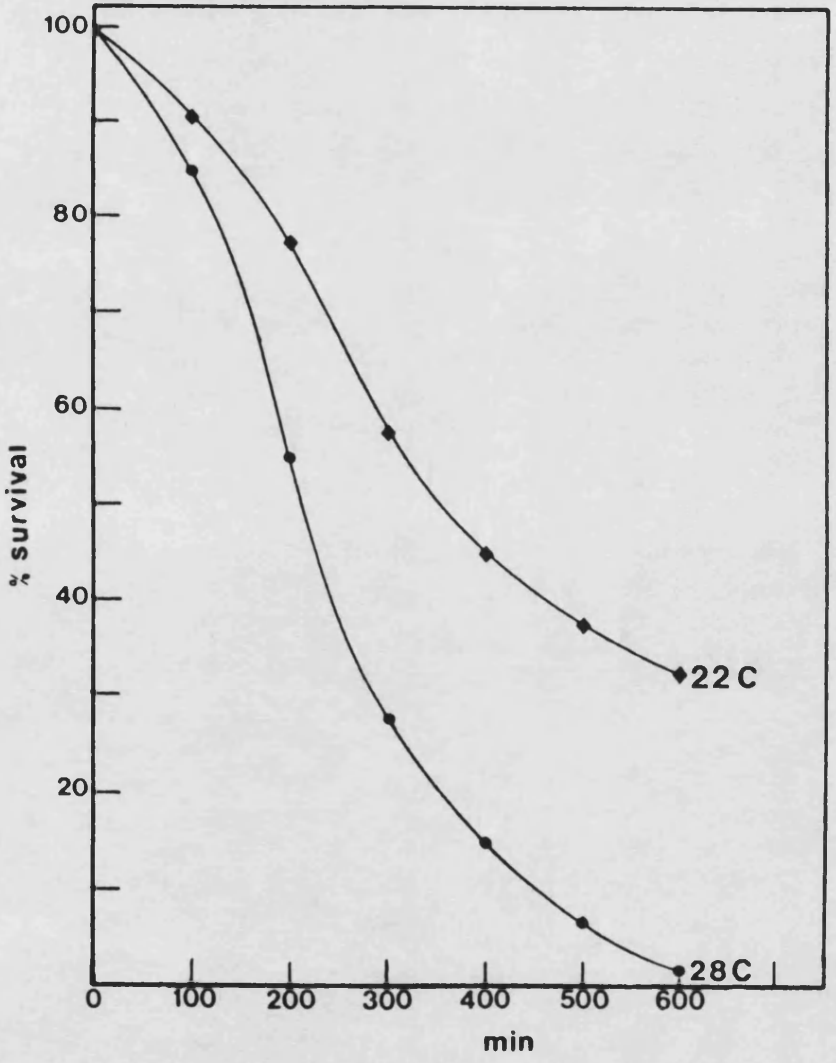
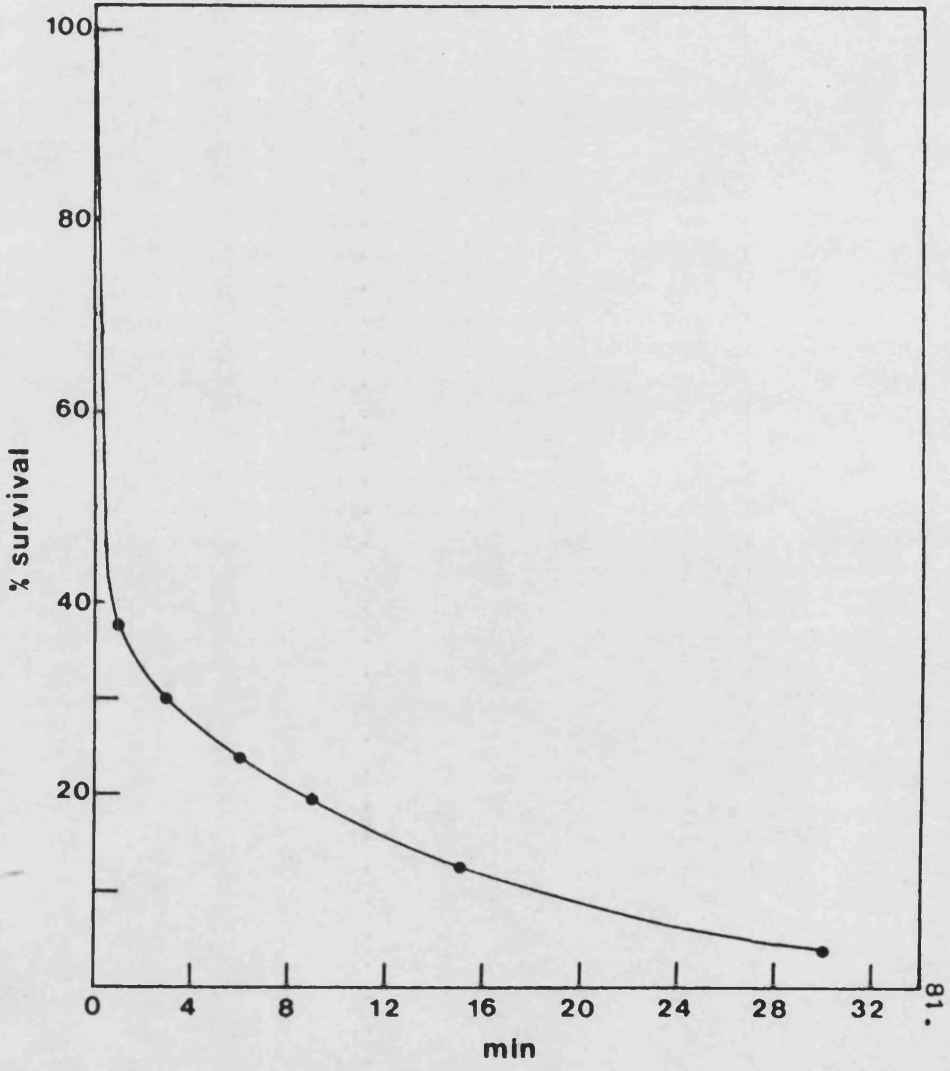

100
80
60
40
20
% survival
22 C
28 C
0
100
200
300
400
500
600
min

Fig 5


100
80
60
40
20
% survival
0
4
8
12
16
20
24
28
32
min

germination of some colonies was delayed, probably as damaged DNA was being repaired. An incubation period of 7.5 h at 28°C was eventually used to obtain a 10 % survival of mutagenised conidia for use in selection experiments.

12.2. *B. allii.*

Spores of *B. allii* begin to germinate within 2 h when immersed in water, thus substantial changes could occur during mutagenesis with EMS, where a long incubation period is necessary. To avoid heterokaryosis and complementation by sister nuclei, which could mask the desired mutants, the alkylating agent *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) was used in place of EMS. NTG is the most powerful mutagen known (Adelberg *et al.*, 1965) and long incubation periods are unnecessary. NTG is highly toxic and carcinogenic and so it was obtained in sealed vials (Isopac, Sigma) containing 10 mg quantities of the chemical, which were then dissolved in acetone by injecting 10 ml acetone through the butyl rubber cap.

1 ml of this solution was added to 9 ml of K phosphate buffer (pH 7.0, 0.05 M) containing 1 x 10^7 spores obtained from 10 d YEP plates of *B. allii* 2070, (final concentration of NTG was 100 $\mu g\ ml^{-1}$). A survival curve (Fig 5) was established as described above for VAA/EMS where sodium thiosulphate was used to quench the NTG (NTG has a $t_{\frac{1}{2}}$ of 15 min in water and is inactivated almost instantaneously in sodium thiosulphate). Spores were dispersed onto 14 cm YEP plates and incubated at 18°C for 4 d.

Spores incubated for 18 min in NTG at 24 °C had a 10 % survival rate.

13. Selection media.

13.1. Polygalacturonase detection media.

The PG detection medium used to isolate PG-deficient mutants of *V.dahliae* (Howell, 1976) was found to be non-specific for PG detection; the pH of this medium, drifted during colony development from pH 5.0 to pH 7.6 (measured with a surface pH electrode), and in such alkaline conditions PL is produced and active. In addition to NAPP, yeast extract and peptone were included which could cause catabolite repression of PG.

The medium described below was based on the inorganic salts media used for PG induction shake flasks with the following alterations:

1) Trace elements were excluded as these tended to precipitate the NAPP.

2) MES was replaced by citrate buffer as the former weakened the gel, reduced the quality of the clearing zones that are revealed on addition of the precipitating agent cetyltrimethyl ammonium bromide (CTAB) and furthermore, failed to keep the pH at 5.0 or below.

3) Oxoid 3 agar was found to cause the NAPP to gel. According to the manufacturer's notes it contains 1700 ppm Ca^{2+} which was the probable cause of the precipitation of the NAPP. Oxoid 1 was used instead as this only contains 150 ppm Ca^{2+}.

The medium was made in two parts which were added together prior to autoclaving (116°C, 10 min).

Part 1: 1 % NAPP w/v was prepared in 250 ml aliquots by adding the powder to distilled water in a high speed Waring blender.

Part 2: To 250 ml citrate buffer (pH 5.0, 0.1 M) 1 g $NaNO_3$, 0.5 g KH_2PO_4, 0.25 g $MgSO_4$ and 7.5 g Oxoid 1 agar were added.

Part 2 was then blended into part 1.

13.2. Pectin lyase detection media.

The medium employed by Hankin & Anagnostakis (1975) contained yeast extract (that could cause CR) and was non-specific for PL detection as the pH drifted on incubation. Howell (1976) used a medium containing sorbose (1 %) and bicene (0.1 M) which was adjusted to pH 9.0 prior to autoclaving. It was found in this study that on autoclaving the medium pH dropped to pH 8.0. During colony development the pH further dropped to 6.8 which would not exclude PG activity. The following medium was developed to enhance PL production while excluding the production of PG.

The medium was made in two parts which were mixed together after autoclaving, because pectin is degraded when heated in alkaline conditions (Albersheim, 1959). Demethylation of the pectin which may also result would reduce the specifity of the substrate; reduction in viscosity reduces the amount of precipitation by CTAB and therefore visualisation of activity.

Part 1: 1 % pectin was prepared as described for NAPP.

Part 2: To 250 ml HEPES buffer (pH 8.0, 0.1 M) 1 g $NaNO_3$, 0.5 g KH_2PO_4, 0.25 g $MgSO_4$ and 10 g of calcium rich Oxoid agar 3 was added.

Part 1 was autoclaved (116°C, 10 min) at pH 4.0 (natural pH of pectin which is only 75 % methylated) and mixed with part 2 after autoclaving (121°C, 15 min), and the pH was then readjusted to 8.0 with 1 M NaOH.

PG and PL activity was detected on these media by the addition of 1 % CTAB, dissolved in distilled water and heated to 30°C. Enzyme activity is apparent as cleared (degraded) zones in the precipitated substrate, which forms a white/grey background.

14. Isolation of mutants.

For selection of mutants, mutagenised spores were spread onto 14 cm

plates of PG or PL media (containing *c* 36 ml media) at *c* 70-150 surviving spores/plate. As CTAB is harmful to fungal growth (Hankin & Anagnostakis, 1975) colonies had to be replicated; therefore sterile 14 cm Whatman # 1 filter papers were placed over the inoculated plates (Fantes & Roberts, 1973), and after 7 d incubation, these and the accompanying inocula from the parent colonies were transferred to fresh media (Czapek Dox for VAA and YEP for *B. allii*). After further incubation (VAA at 23°C, *B. allii* at 18°C) discrete colonies became established on these media and conidia formed on the surface of the filter paper. Marks were made on the lower side of the detection plates with water proof ink to indicate individual colonies before the plates were flooded with CTAB and incubated at room temperature (*c* 5 min) until cleared zones became apparent. Colonies that lacked or had reduced or increased zones of clearing were noted. Isolates of putative mutants were removed with a sterile needle from the replica plates and inoculated onto Czapek Dox or YEP to select against auxotrophic and metabolic mutants. Surviving colonies were screened once more on solid selection media before further critical study.

15. Mutant testing *in vitro*.

Of the putative mutants selected by the procedures outlined above, three isolates with altered capabilities to produce PL, were studied in depth to test for further unwanted mutations or pleiotropic effects and furthermore to confirm alteration in enzyme production or secretion. Replication was made in each test so that an analysis of variance could be made between the strains.

15.1. Morphology.

Colony and spore morphology was examined in liquid and solid media.

15.2. Growth.

15.2.1. Liquid culture.

+Type and mutant strains were grown in shake flasks on buffered pectin (0.5 % at pH 5 and 8), GALA (0.5 %) and glucose (0.5 %) for 7 d as described in Materials and Methods 1. Final mycelial dry weights were then determined.

15.2.2. Spore production.

Spore production of strains grown on host cell walls, pectin and glucose was determined with an improved Neubauer haemocytometer.

15.2.3. Radial growth.

Radial growth of colonies developing from 8 mm plugs taken from the edge of 10 d old Czapek-Dox plates was measured on alternate days for 16 d.

15.2.4. Enzyme production.

15.2.4.1. PL production on host cell walls.

PL production by these strains grown on host cell walls (0.2 %) and pectin (0.5 %, pH 8 and 5 respectively) was studied over 7 d.

15.2.4.2. PG, cellulase, β-D galactosidase, β-D glucosidase and L-leucine arylaminidase.

The activities of these enzymes were all assayed in filtrates of cell wall cultures. Enzyme activities were also determined by API-ZYM.

15.2.4.3. Intracellular PL activity.

Extracellular and intracellular PL activities were determined in culture filtrates and mycelial extracts of +Type and mutant strains grown for 7 d in PL inducing media (pectin 0.5 % w/v, pH 8.0). Alkaline phosphatase activity was used as an intracellular marker.

Mycelial extracts were prepared following methods for obtaining hyphal cell walls (Lisker & Retig, 1975; Polacheck & Rosenberger, 1975).

Mycelium from three shake flasks was collected on Whatman # 1 filter paper by Buchner filtration. The culture filtrate was dialysed and frozen at -20°C. The mycelium was retained on the Buchner funnel and washed in 4 x 100 ml cold HEPES buffer (pH 8.0, 0.05 M, 4°C) to remove extracellular enzymes; it was then comminuted in 20 ml cold buffer for 5 min in an overhead homogeniser, with cooling, in an ice bucket. The mycelial suspension was made up to 50 ml in similar buffer and ultrasonicated for 45 min (75 KW and 20 KC) until only broken hyphal walls could be seen under the microscope. The suspension was kept at 10 °C with a water jacket during ultrasonication. After centrifugation (23 000 x g, 15 min, 4°C) the supernatant was dialysed and frozen.

Filtrates were assayed for PL and α and β alkaline phosphatases.

16. Mutant testing *in vivo* - evaluation of pathogenicity and virulence.

16.1. *V. albo-atrum.*

5-6 week old tomato plants (Craigella GCR 26) arranged in a random block design were inoculated with 50 ml 0f 3 d old conidial suspensions (1×10^6 ml^{-1}) from cultures (filtered through 4 layers of muslin) of +Type and mutant strains grown on 0.5 % glucose in shake flasks. Conidial suspensions were poured onto the soil without causing root damage. The plants were maintained in a temperature controlled greenhouse at 20 (±1°C) as this is considered to be optimal for VAA

infection of tomato (Cooper, 1974). Epinasty, chlorosis, necrosis and symptoms of water stress were noted periodically as they developed. The extent of disease in each plant was quantified by the number of lowest 8 true leaves that were affected by each symptom. The data was analysed by a two way analysis of variance without replication.

At the end of each experiment the extent of colonisation of the vascular system was estimated. Hand sections were taken with double edged razor blades at the mid point of the first, third, fifth and seventh internodes. These were mounted in 0.01 % cotton blue and lactophenol. Host colonisation was evaluated by assessing the percentage number of vessels containing hyphae (Cooper & Wood, 1980).

+Type and mutant strains were reisolated from the infected plants. Sections of stem were taken at intervals along the stems and surface sterilised in 10 % chloros and 0.03 % Tween 80 for 5 min. The segments were then placed onto Czapek-Dox plates and incubated at 23°C for 3-7 d. Presence or absence of VAA was noted and the amount of fungal outgrowth was estimated visually.

+Type and mutant strains reisolated by the above method were compared with stock cultures to ensure that they had retained their phenotypic characteristics.

16.2. *B. allii.*

Succesful colonisation of onion tissue was determined as the ability for a spreading lesion to form at the site of inoculation at the rate of 2×10^4 (1×10^6 conidia ml^{-1}) in a 20 µl droplet, (Stewart & Mansfield, 1985a). Mid-bulb scales were cut into 2 x 3 cm sections and placed in 14 cm petri dishes lined with moist filter paper. Inoculum droplets were applied to the inner epidermis *via* a Finnpipette. The inoculated tissue was incubated at 18°C. Lesions developed after 3 d.

17. Histological techniques.

Light microscopy. Black and white and colour photomicrographs were taken on an Olympus Photomicrograph System (PM-10 ADS).

17.1. Fungi.

Spores and mycelium associated with plant material were distinguished by staining with 0.1 % cotton blue in lactophenol.

17.2. Pectin.

Pectin degradation was detected by staining plant tissue with 0.05 % ruthenium red . Degraded walls were thicker and stained more intensely.

17.3. Host cell viability.

Viability of onion cells in infected or pectinase-treated tissue was determined by vital staining with neutral red (Tribe, 1955). Epidermal strips (5 x 5 mm) cut from the inner side of mid-bulb scales with a scalpel were plasmolysed in 0.001 % neutral red and 1 M KNO_3 in Na phosphate buffer (pH 8.0, 0.01 M). Viable cells retained neutral red within vacuoles.

17.4. Esterase activity.

Esterase activity associated with *B. allii* hyphae growing on onion epidermis was directly detected by the α-naphthyl ester method (Gomori, 1950). 10 mg α-naphthyl propionate (C_3) in 0.35 ml acetone was added to 20 ml K phosphate buffer (pH 7.0, 0.1 M) and shaken vigorously until most of the cloudiness had disappeared. 75 mg fast blue BB was added, shaken, and after filtration the reagent was added to preparations of infected onion epidermis. After incubation for 1-15 min at room temperature, the epidermal strips were thoroughly washed with distilled

water and immediately observed. A violet colouration indicated a positive result.

17.5. Fluorescence staining of fungal nuclei.

Fungal spores from 10 d old plates were fixed in 5 % glutaraldehyde for 30 min. A portion of the suspension was diluted with an equal volume of buffered (pH 7.0, Tris, 0.05 M) *4,6*-diamidino-*2*-phenylindole (DAPI) 1.5 µg ml^{-1}, (Hooley, Fyfe, Maltese & Shaw, 1982). After 2 h incubation at 30°C spores were observed with a Leitz Orthoplan Wetzlar microscope fitted with an excitation filter (390 - 450 nm transmission). Fluorescence occurs at 450 nm.

18. Effect of pectinases on intact host tissue.

Squares (5 x 5 mm) of inner epidermis were cut with a scalpel from mid-bulb onion scales and washed in two changes of deionised water. 25 squares were placed in universals and incubated upto 48 h at 20°C in the following conditions:

1) 5 ml PG (activity 500 RVU ml^{-1}) from IEF fraction 9 (pI 5.4) in MES buffer (pH 5.0, 0.1 M).

2) 5 ml PL (activity 0.21 µmol UGAL ml^{-1} min^{-1} per ml solution) from IEF fraction 14 (pI 7.2) in HEPES buffer (pH 8.0, 0.1 M).

3) 5 ml containing all the PG isoenzymes and PL from pectin inducing media (same total activities as the two previous independent solutions) in HEPES buffer (pH 7.0, 0.1 M).

Autoclaved enzymes were used in control treatments. The epidermal squares were gently rotated to ensure good enzyme contact. Enzyme activity in the supernatant was assayed at 12 and 48 h.

The effect of the enzymes on tissue was assessed in the following ways:

1) Cell killing. Strips were periodically removed from the solutions and stained with neutral red by the method outlined above. The number of surviving cells was estimated by taking the mean percentage of cells that plasmolysed and retained dye in 4 fields of view on two strips.

2) Cell leakage was estimated by determining the release of K^+ from the tissue into the surrounding solution. 200 µl samples were periodically removed and frozen at -20°C. The samples were made up to 2 ml with deionised water and the K^+ concentration was determined with a Corning flame photometer.

3) Macerating activity was estimated by testing the loss in coherence of the treated squares by gently teasing them between two dissecting needles (Cooper *et al.*, 1978). A macerating index (MI) from 0 to 4 was used where:

0	control tissue
1	
2	↓
3	no resistance
4	coherence lost, collapses on picking up.

Macerating activity, ion loss and the neutral red test could all be conducted for replicate tissue squares in the same treatment.

19. Chemicals.

All chemicals were of analytical grade unless otherwise stated and were supplied by Sigma or BDH Ltd.

RESULTS AND DISCUSSION

The aim of this study was to obtain mutants of *B. allii*, *C. lindemuthianum and V. albo-atrum* that are altered in their ability to produce PG and PL. The selection procedure relies on detecting the enzymes which are induced in colonies grown on a solid pectic agar medium (Material and Methods 13). To confirm that the regulation of PG and PL production by an isolate of VAA was similar to that determined by Cooper & Wood (1975), enzyme activity was determined in filtrates from cultures of the Wild Type containing different carbon sources.

Similarly, as previous workers have not considered the effects of pH, CR or substrate in cultures of *Botrytis* species, the regulation of PG and PL production by *B. allii* was studied in detail. PG and PL production was also examined in established cultures of VAA and *B. allii* in the presence or absence of cycloheximide, which inhibits the synthesis of protein, to determine whether the enzymes were produced by *de novo* synthesis in the presence of inducing substrate.

PG and PL production were also monitored in onion tissue inoculated with *B. allii* conidia. Although high levels of PL have been found in VAA infected cuttings and stem segments of tomato, many attempts at isolating CWDE from whole tomato plants have failed or given inconsistent results (Cooper & Wood, 1980; Matta & Dimond, 1963). Therefore no further attempt was made here to determine the levels of pectinase produced *in vivo* by VAA.

The properties of PG and PL were also studied in detail as these may offer some insight into their rôle in disease. These are reported in Results and Discussion 2. In addition, the selection of mutants, deficient in PG or PL relies on the contrasting properties of the enzymes under different pH and ionic conditions.

Section 3. is concerned with the selection, characterisation and pathogenicity testing of pectinase mutants.

1. Regulation of polygalacturonase and pectin lyase production by *V. albo-atrum* and *B. allii in vitro* and *in vivo*.

1.1. Production of PG and PL on different carbon sources.

1.1.1. *V. albo-atrum*.

VAA was grown on 8 different media. Pectin (or NAPP) was used to induce the synthesis of PG and PL; glucose was added to pectin (or NAPP) to repress production of the enzymes in the presence of an inducing polymer; glucose was also used as sole carbon source to provide non-inducing, repressing conditions for determining basal production; carboxymethyl cellulose (CMC) was employed to determine basal or repressible constitutive production, as this polymer should not cause CR or induce pectinases. Media were buffered at pH 4-5 (MES) or 8.0 (HEPES) as PG and PL production are favoured in acidic and alkaline conditions respectively (Cooper & Wood, 1975). Shake flasks containing 200 ml media were inoculated with 1 x 10^7 conidia and incubated for 13 d on a rotary incubator (25°C, 150 rpm). Samples were removed from the flasks during the incubation period and assayed for PG, PL, glucose (where applicable) and pH.

Table 5 shows the final pH, mycelial dry weights and conidial numbers of the cultures. Mycelial growth and conidial production were generally greater in alkaline conditions and were most luxuriant on glucose alone or combined with NAPP. Pectin supported moderate growth; conidial production and growth was poorest on CMC. Growth in the pectin/glucose cultures was 3 fold less that in NAPP/glucose at pH 8.0 and may have

Table 5.

Growth and production of PG and PL on different carbon sources by V.albo-atrum; maximum PG and PL activities attained during 13 d incubation at 25 C on a rotary incubator (150 rpm).

Carbon source and buffer pH	final pH	PG (RVU) a	PL ($\mu g\ ml^{-1}h^{-1}$) b	dry weight (mg) c	conidia (ml^{-1}) d	maximum activity mg^{-1}final mycelial dry weight PG e	PL f
Pectin, pH 4.5	6.0	3965.7	60.0	408	6.42×10^5	9.72	0.147
NAPP, pH 8.0	8.2	154.5	422.5	358.25	1.48×10^8	0.4313	1.179
Pectin/glucose, pH 4.5	6.0	7.3	0	464.5	$< 10^4$	0.0517	-
NAPP/glucose, pH 8.0	8.1	4.7	12.5	1208.3	1.21×10^8	0.0039	0.0103
Glucose, pH 4.5	6.25	19.6	0	1507.6	7.95×10^7	0.0130	-
Glucose, pH 8.0	8.0	1.4	0	1587.0	1.83×10^8	0.0009	-
CMC, pH 4.5	5.1	10.0	0	183.75	1.75×10^4	0.0567	-
CMC, pH 8.0	8.0	2.5	0	253.15	2.12×10^6	0.0098	-

a PG activity determined viscometrically.
b PL activity determined by TBA.
c Mycelium dried on preweighed dried Whatman 1 filter paper at 70 C to constant dry weight.
d Conidial density determined on an improved Neubauer haemocytometer.
e Arbitary figure derived by dividing the maximum PG activity attained during the time course by the final mycelial dry weight.
f Arbitary figure derived as for (e) using the maximum PL activity.

All carbon sources supplied at 1 % (w/v) except glucose which was added at 3 % .

Figs 6-13. Production of polygalacturonase and pectin lyase in *V. albo-atrum* cultures containing different carbon sources in acidic and alkaline conditions.

Fig 6. Pectin (1 % w/v; pH 4.5, MES 0.05 M).

Fig 7. NAPP (1 % w/v; pH 8.0, HEPES 0.05 M).

Fig 8. Pectin (1 % w/v) and glucose (3 % w/v); pH 4.5, MES 0.05 M).

Fig 9. NAPP (1 % w/v) and glucose (3 % w/v); pH 8.0, HEPES 0.05 M).

Fig 10. Glucose (3 % w/v; pH 4.5, MES 0.05 M).

Fig 11. Glucose (3 % w/v; pH 8.0, HEPES 0.05 M).

Fig 12. CMC (3 % w/v; pH 4.5, MES 0.05 M).

Fig 13. CMC (3 % w/v; pH 8.0, HEPES 0.05 M).

Shake flasks containing 200 ml media were inoculated with 1 x 10^7 conidia and incubated on a rotary incubator (25°C, 150 rpm). PG (◇) assayed viscometrically, activity expressed as RVU. Pectin lyase (◆) assayed by TBA, activity expressed as $\mu g\ ml^{-1}\ h^{-1}$ UGALA released. Glucose $mg\ ml^{-1}$ (■) determined by glucose oxidase assay; pH (△). Mean results of 4 cultures.

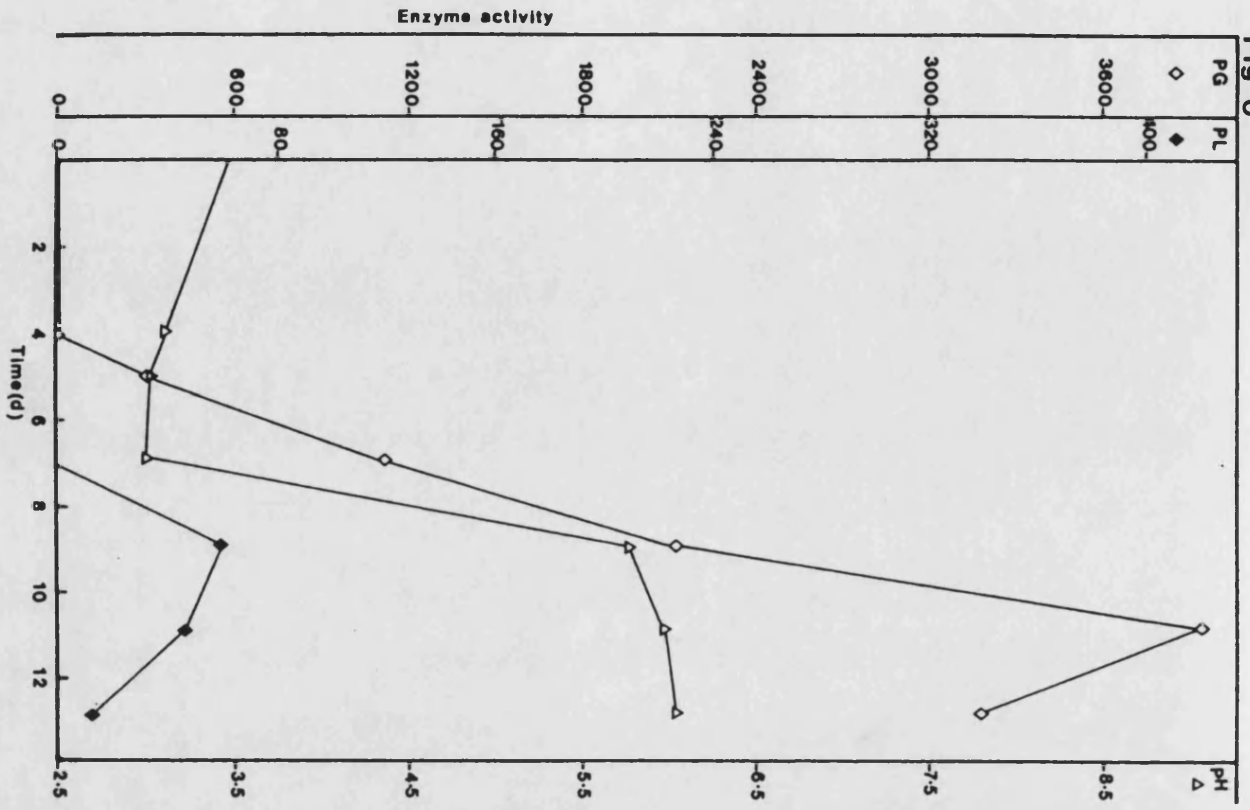

Enzyme activity
PG
PL
pH
Time(d)

Fig 6

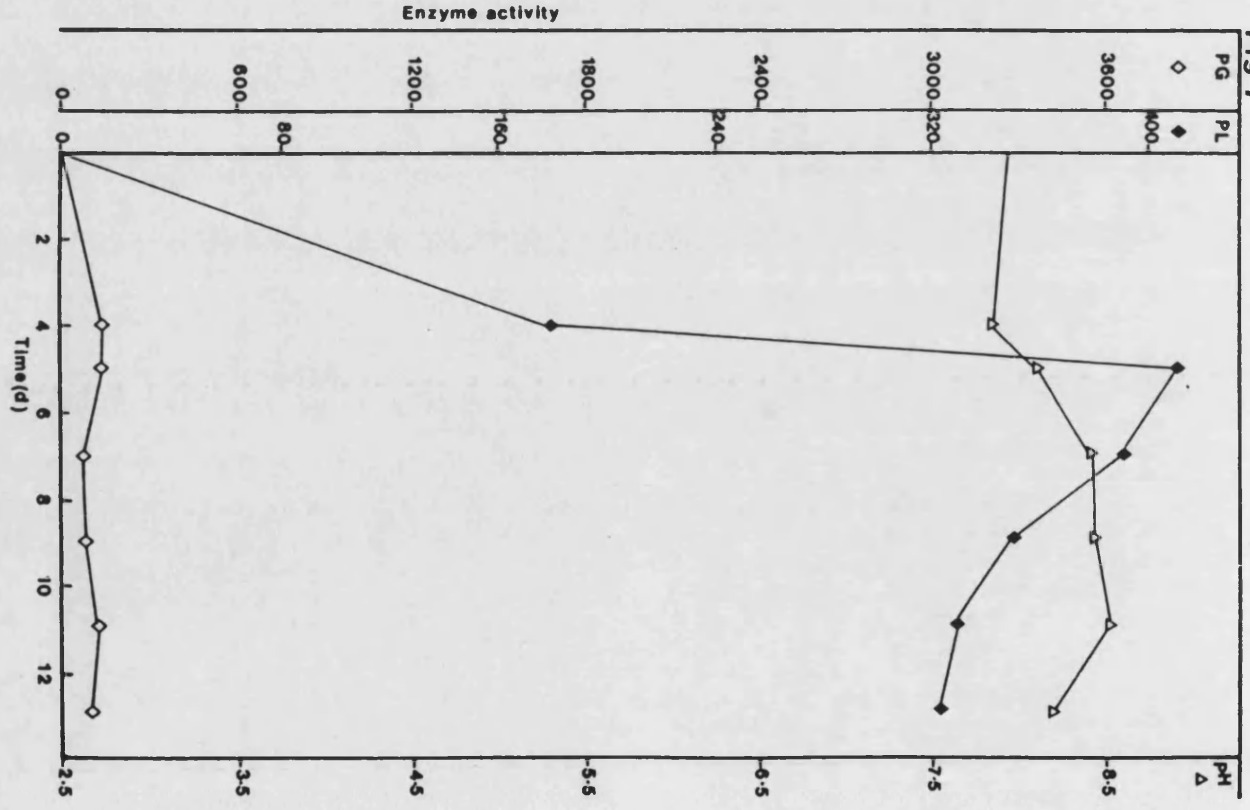

Enzyme activity
PG
PL
pH
Time(d)

Fig 7

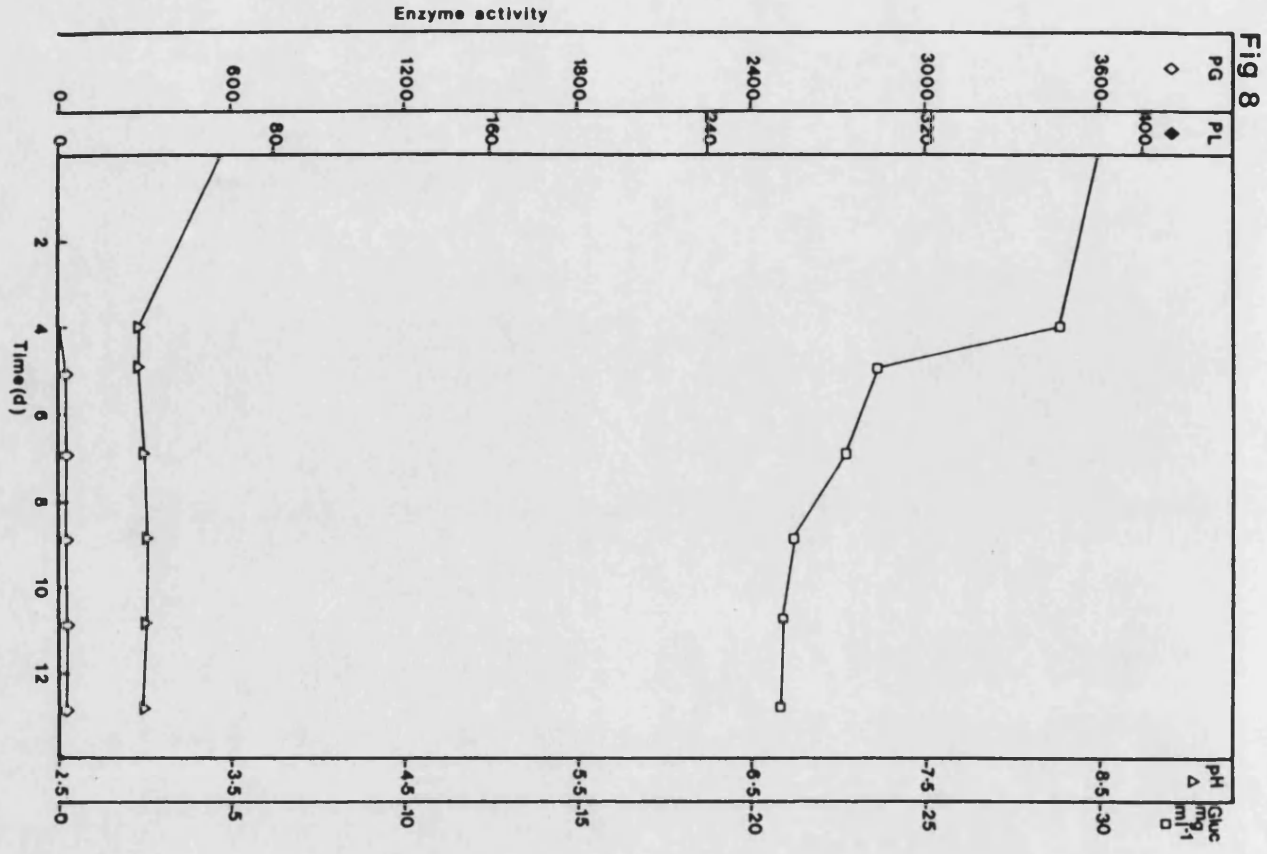

Enzyme activity
PG
PL
pH
Gluc mg ml-1
Time(d)

Fig 8

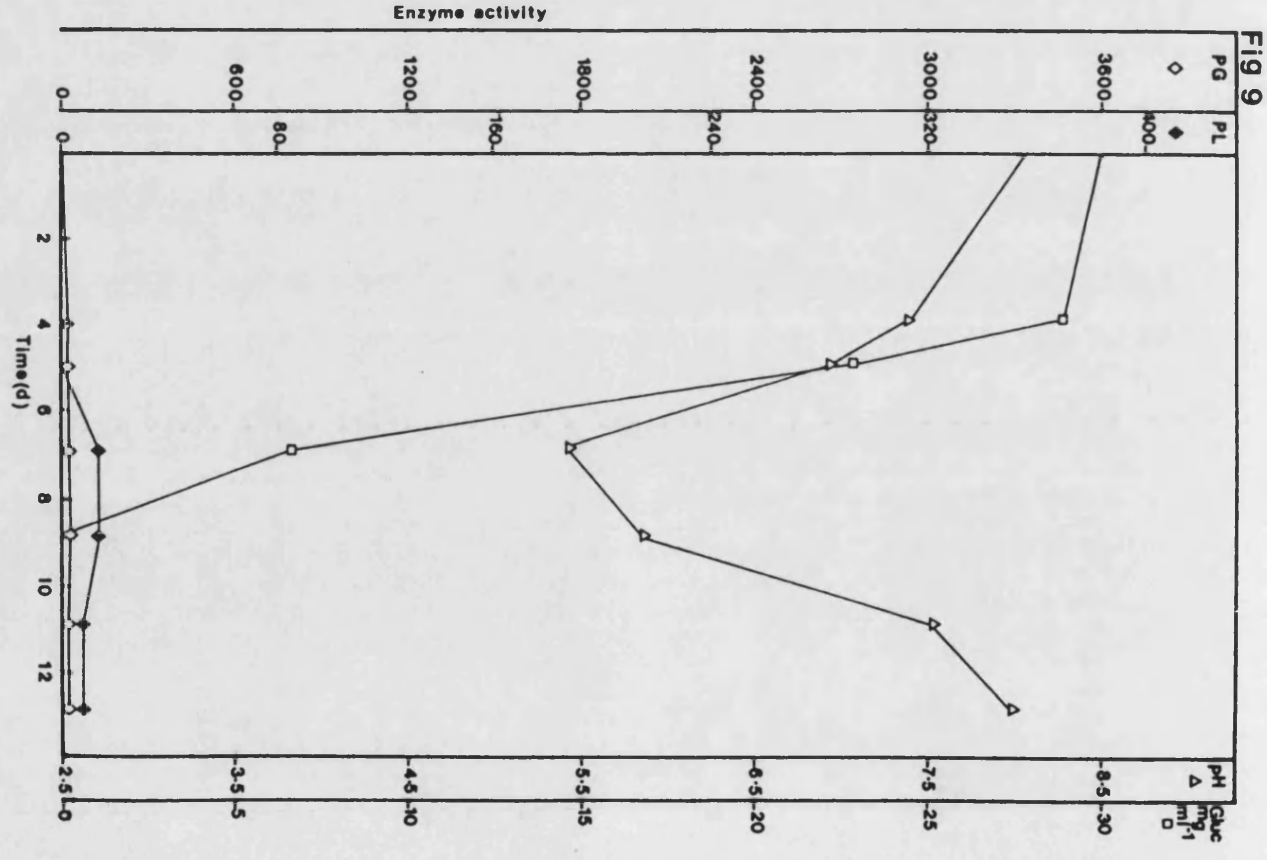

Enzyme activity
PG
PL
pH
Gluc mg ml-1
Time(d)

Fig 9

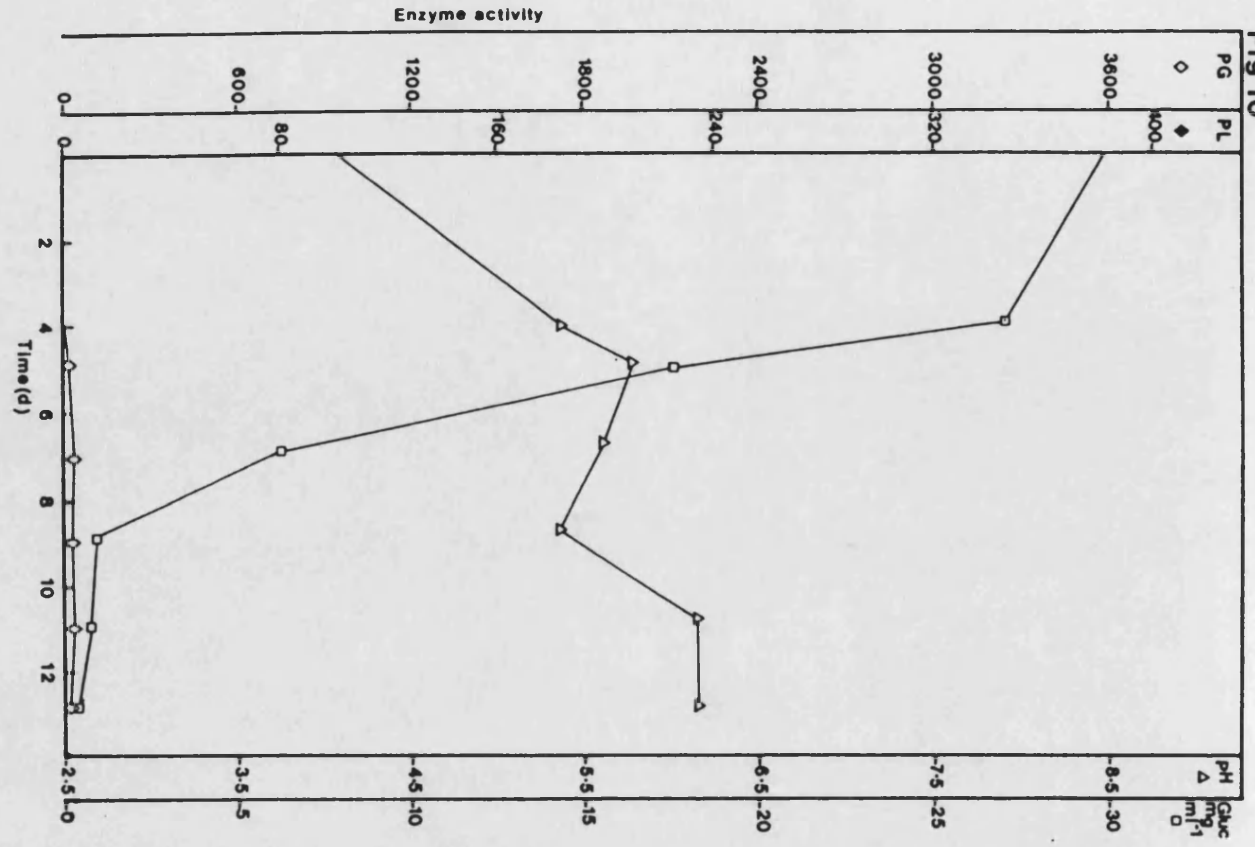

Enzyme activity
PG
PL
Time(d)
pH
Gluc
mg ml-1

Fig 10

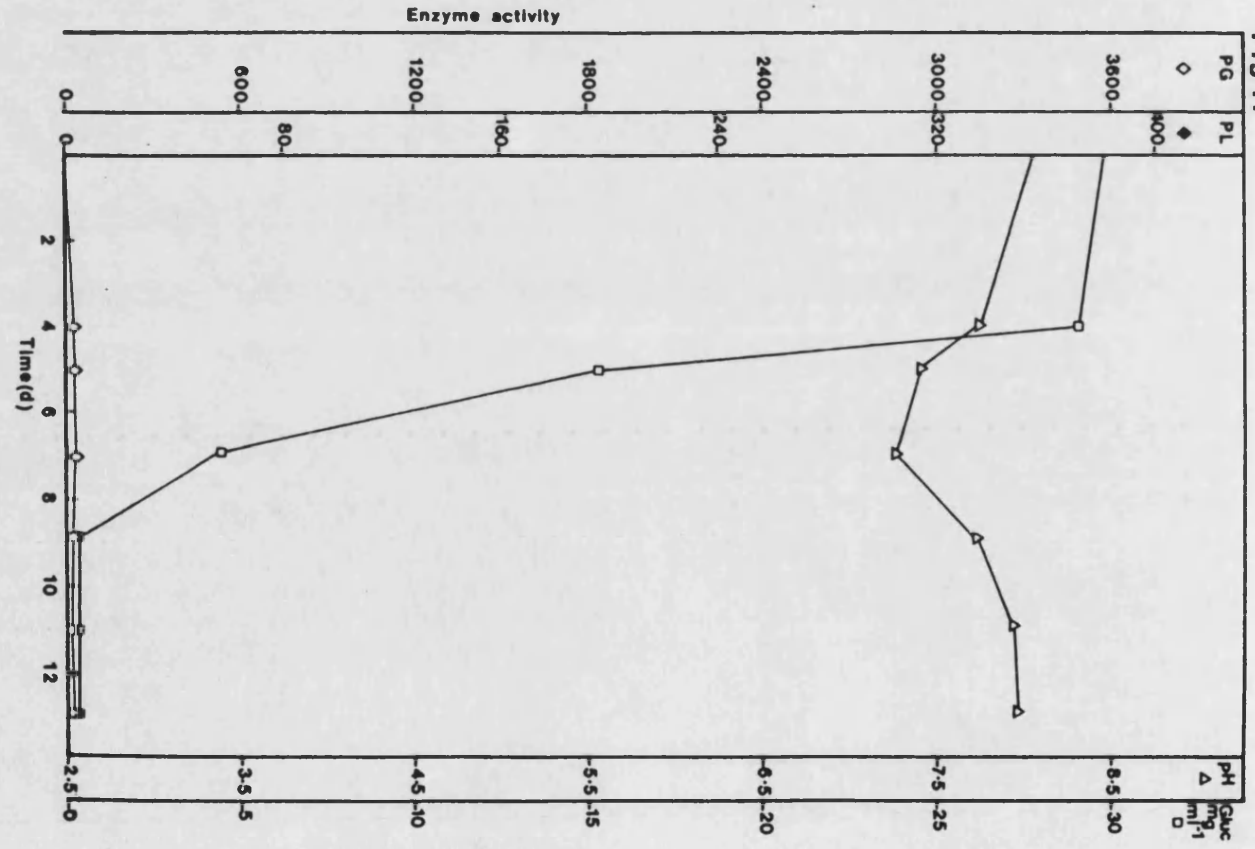
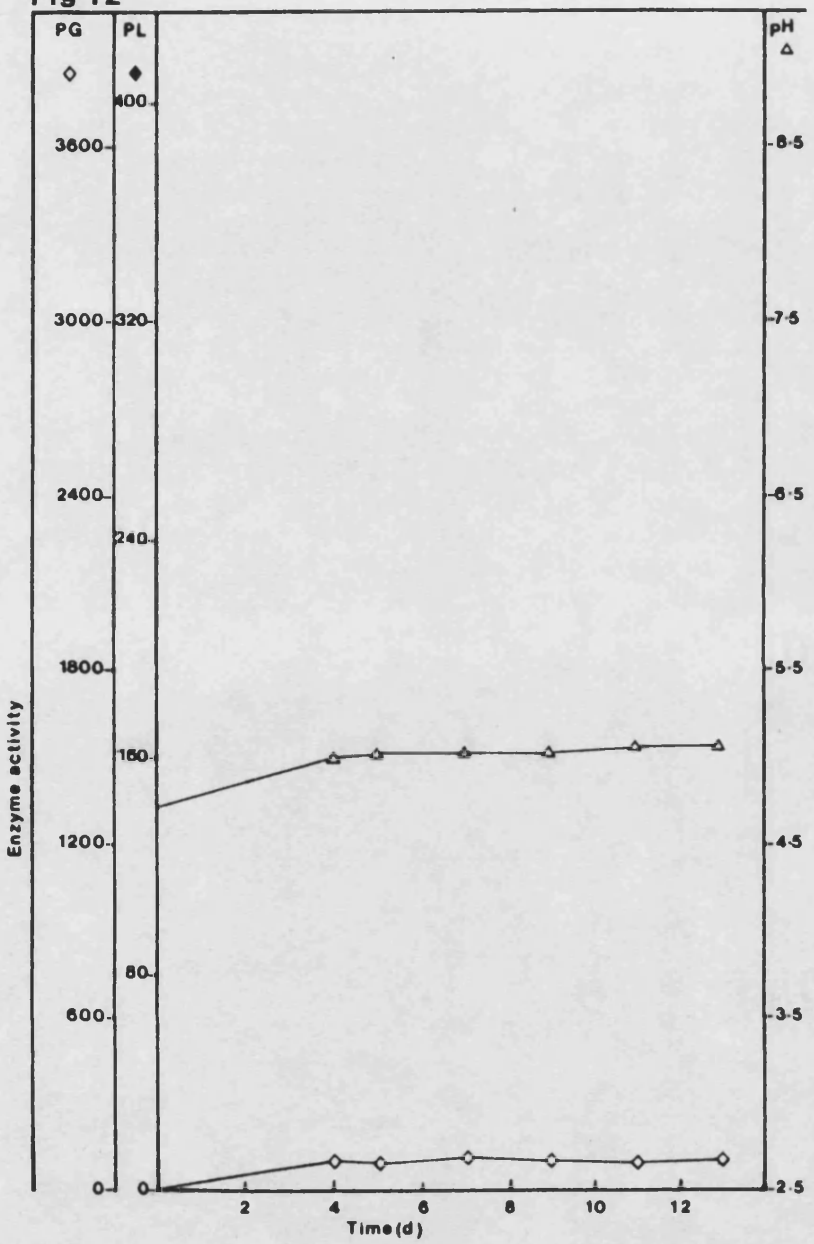

Enzyme activity
PG
PL
Time(d)
pH
Gluc
mg ml-1

Fig 11

Fig 12


PG
PL
pH
Enzyme activity
Time(d)
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600
1200
1800
2400
3000
3600
80
160
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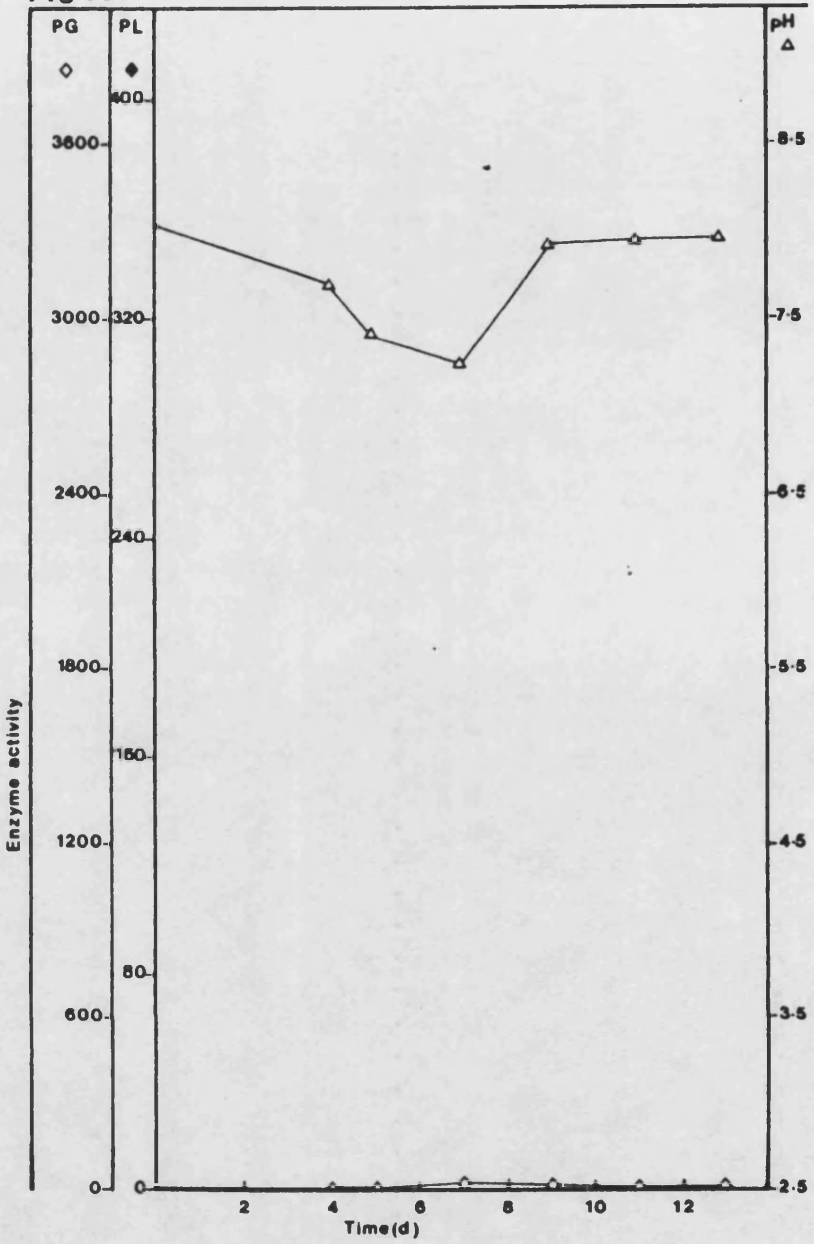
12

Fig 13


PG
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6.5
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12

been retarded because the pH failed to rise as it had done in cultures containing pectin alone. 2-7 fold more carbon was provided for the VAA and thus growth was duly 5-10 fold greater than in the *B. allii* cultures (Results and Discussion 1.1.2.). However, when this is taken into account growth by VAA was comparatively better (Tables 5 and 6).
PG was detected at very low levels on CMC and glucose, which was characteristic of basal production. PL was not found in glucose or CMC cultures even following a 200 fold concentration of the filtrates. PG and PL were both induced on NAPP and pectin, but the relative amounts that were produced were dependent on the pH. Acidic conditions favoured PG production which was 25 fold greater than under alkaline conditions, whereas PL was produced maximally in alkaline conditions, and was only detected in acidic pectin media after 9 d, when the pH had drifted from below 3.5 to 5.75. The pH of the acidic pectin medium was initially low as it fell to 3.5 during autoclaving. In later experiments, with *B. allii*, media pH was adjusted to 5.0 with 1 M NaOH prior to inoculation.
The presence of glucose caused CR and prevented induction of PG and PL, although growth was vigorous on media containing both polygalacturonan and glucose (Fig 9 and Table 5). PL and PG were only detected after the glucose levels had declined to below 5 $\mu g\ ml^{-1}$, but by this time (7 d), the mycelium was already extensively autolysed, (determined microscopically), and thus PL synthesis remained minimal.
Under optimal conditions PL production peaked at 5 d, but PG levels reached a maximum after 11 d.

1.1.2. *B. allii.*

Growth, pH, GALA and PG/PL production were critically assessed because previous workers have not fully demonstrated the nature of regulation (Mankarios & Friend 1980; Hancock *et al.*, 1964a).

B. allii was grown in shake flasks containing 10 different carbon sources for 14 d on a rotary incubator (25°C, 150 rpm) (Table 6 and Figs 14-23). Growth was visually assessed during incubation and quantitatively estimated as final dry weight flask^{-1} (*B. allii* did not produce blastospores/conidia in liquid culture which precluded continuous assessment of growth). 4 ml samples were removed periodically from the cultures and assayed for PG, PL, pH, and glucose (where applicable).

Similar growth apparently occurred on the different carbon sources in acidic and alkaline conditions although the final dry weights revealed that growth was generally better in alkaline cultures. Growth was first apparent when tiny pellets formed from entangled germ tubes which appeared 24 h after inoculation (1 x 10^7 spores flask^{-1}). After 48 h, growth on pectin/glucose was clearly greater than on pectin alone or on the other substrates (see Appendix 2 for detailed results). Growth on cell walls occurred as small aggregates of fungus around cell wall particulates; after 4 d larger aggregates had also formed. By 10 d all host cell material was either solubilised or bound to the fungal mycelium. Mycelium in all the cultures was extensively autolysed and melanised after 10 d incubation and growth by this time was minimal.

PG and PL were produced at very low levels on CMC and glucose indicating that neither enzyme is produced constitutively; furthermore the lack of enzyme activity on glucose was not merely due to CR because synthesis on CMC (in conditions that were non-repressing), was also minimal (Fig 18 and 20). PG and PL of *B. allii* like those of VAA were induced on pectin and cell walls (Figs 14, 15, 19 and 13). Similarly PG production by *B. fabae* was induced on pectin (Appendix 3) and host cell walls (550 RVU after 7 d incubation at 25°C, 150 rpm). Moreover pH had similar effects on production by the three pathogens; PG and PL

Table 6.
Growth and production of PG and PL on different carbon sources by B. allii; maximum PG and PL activities attained during 14 d incubation at 25 C on a rotary incubator (150 rpm).

Carbon source and buffer pH	final pH	PG (RVU) a	PL (μmolml^{-1}min^{-1}) b	dry weight (mg) c	maximum activity mg^{-1}final mycelial dry weight PG d	PL e
Pectin, pH 5.0	5.8	347.2	0.0026	71.3	4.869	3.65×10^{-5}
Pectin, pH 8.0	6.5	183.3	0.0203	70.5	2.6	2.89×10^{-4}
Pectin/glucose, pH 5.0	5.9	398.0	0.0022	124.25	3.20	1.77×10^{-5}
Pectin/glucose, pH 8.0	5.9	403.0	0.0300	138.5	2.91	2.17×10^{-4}
Glucose, pH 5.0	5.2	44.2	0	71.45	0.621	-
Glucose, pH 8.0	6.2	16.7	0.007	141.1	0.118	4.96×10^{-5}
CMC, pH 5.0	5.2	12.0	0	31.2	0.385	-
CMC, pH 8.0	8.0	4.0	0	50.25	0.079	-
Onion cell walls, pH 5.0	5.2	1420.0	0.0007	-	-	-
Onion cell walls, pH 8.0	7.5	21.0	0.0165	-	-	-

a PG activity assayed viscometrically; b PL activity assayed by UV absorbance at 238 nm.
c Mycelium dried on preweighed Whatman 1 filter paper at 70 C to constant dry weight.
d Arbitary figure derived by dividing the maximum PG activity attained during the time course by the final mycelial dry weight.
e Arbitary figure derived as for (d) using the maximum PL activity.
All carbon sources provided at 0.5 % (w/v) except glucose in the pectin/glucose cultures which was added at 1 % (w/v).

Figs 14-23. Production of polygalacturonase and pectin lyase in *B. allii* cultures containing different carbon sources in acidic and alkaline conditions.

Fig 14. Pectin (0.5 % w/v; pH 5.0, MES 0.05 M).

Fig 15. Pectin (0.5 % w/v; pH 8.0, HEPES 0.05 M).

Fig 16. Pectin (0.5 % w/v) and glucose (0.5 % w/v; pH 5.0, MES 0.05 M).

Fig 17. Pectin (0.5 % w/v) and glucose (0.5 % w/v; pH 8.0, HEPES 0.05 M).

Fig 18. Glucose (0.5 % w/v; pH 5.0, MES 0.05 M).

Fig 19. Glucose (0.5 % w/v; pH 8.0, HEPES 0.05 M).

Fig 20. CMC (0.5 % w/v; pH 5.0, MES 0.05 M).

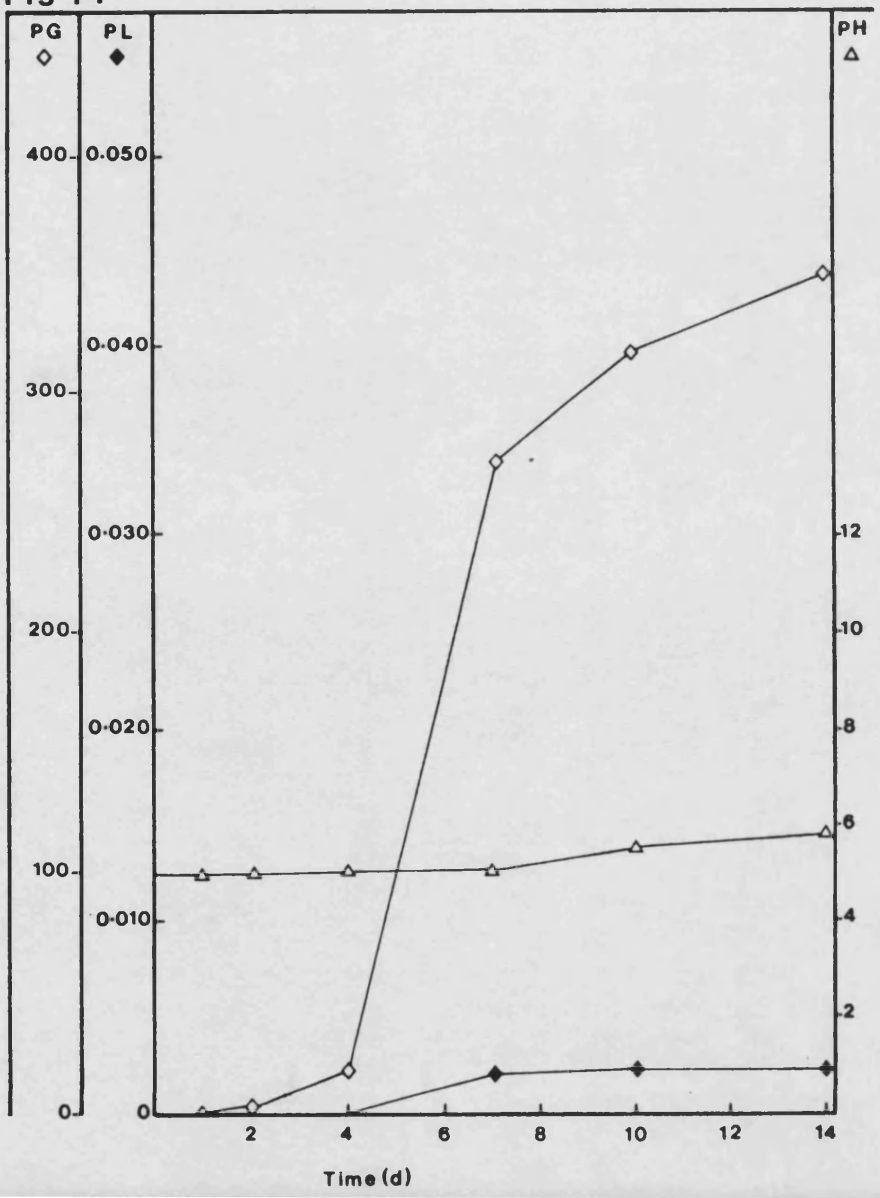
Fig 21. CMC (0.5 % w/v; pH 8.0, HEPES 0.05 M).

Fig 22. Onion cell walls (0.5 % w/v; pH 5.0, MES 0.05 M).

Fig 23. Onion cell walls (0.5 % w/v; pH 8.0, HEPES 0.05 M).

Shake flasks containing 100 ml media were inoculated with 1 x 10^7 conidia and incubated on a rotary incubator (25°C, 150 rpm). PG (◇) assayed viscometrically, activity expressed as RVU. PL (◆) assayed by measuring increase in absorbance at 238 nm, activity expressed as μmol ml^{-1} min^{-1}. Glucose mg ml^{-1} (■) determined by glucose oxidase assay; pH (△). Mean results of 4 cultures.

Fig 14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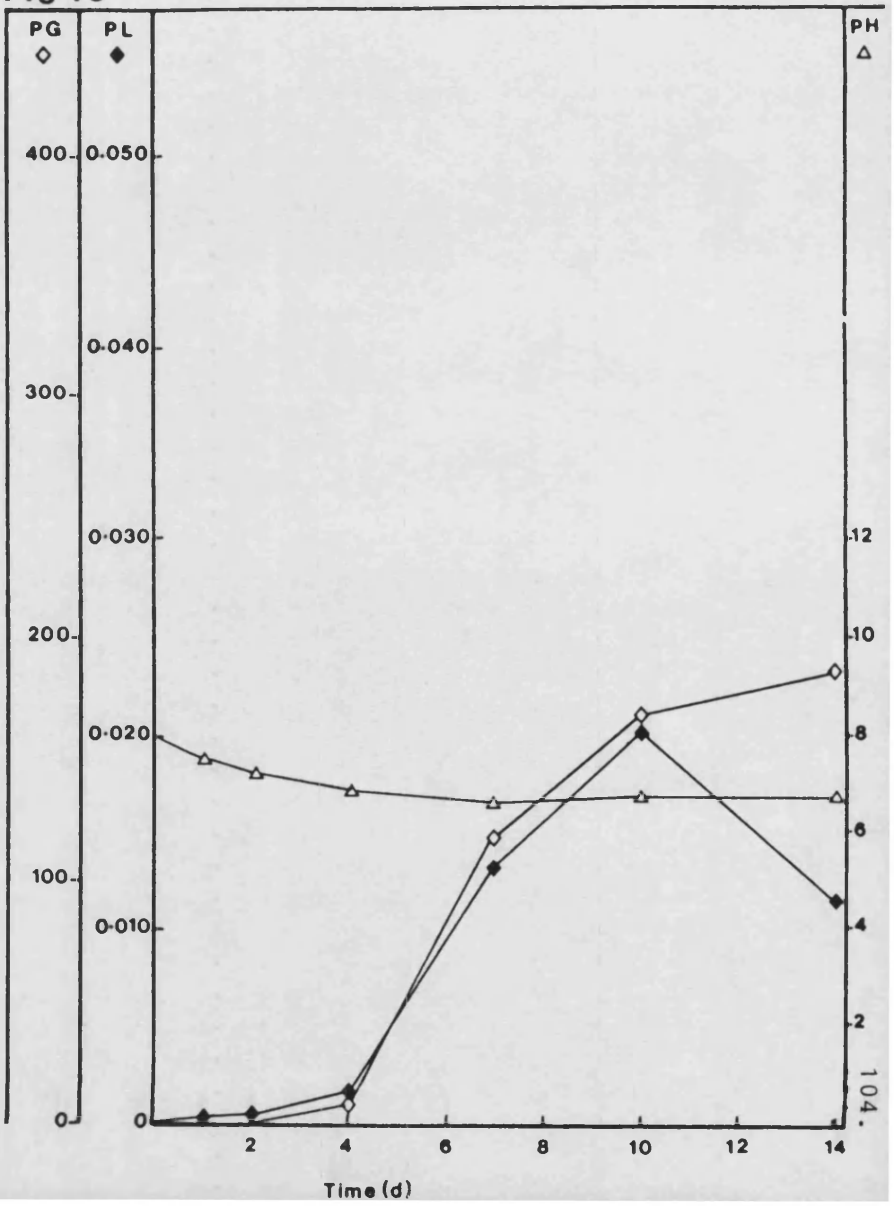
14
Time (d)

Fig 15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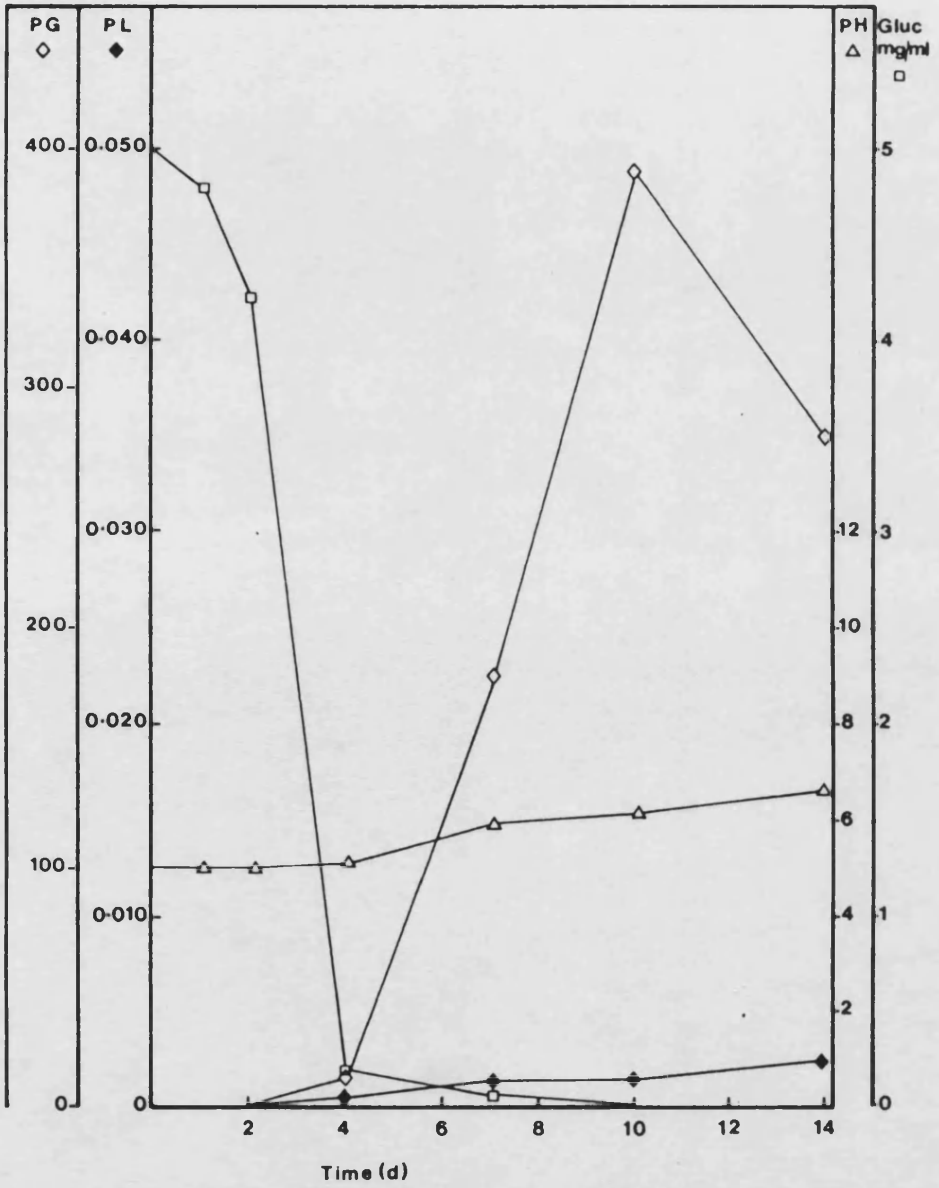
Time (d)

Fig 16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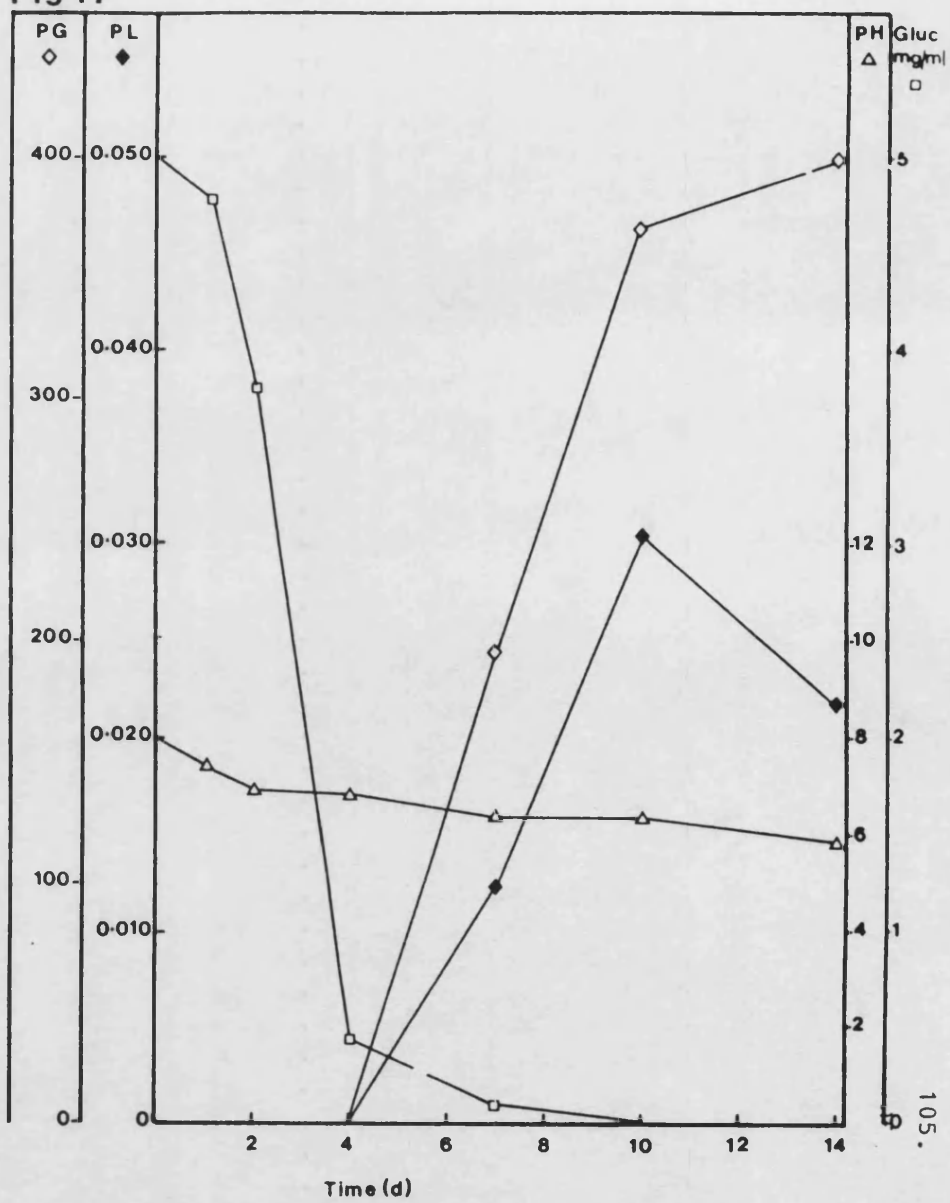
Time (d)

Fig 17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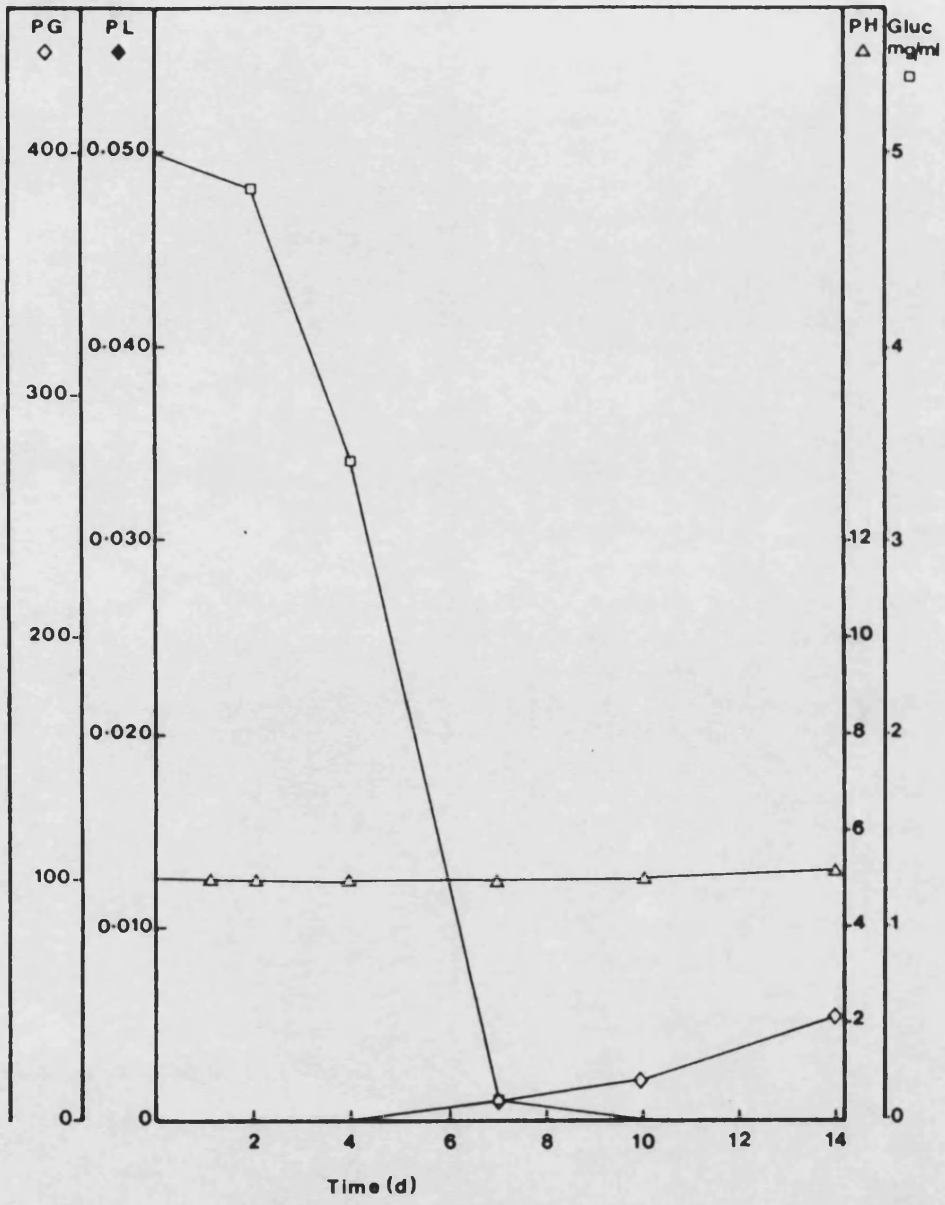
Time (d)

Fig 18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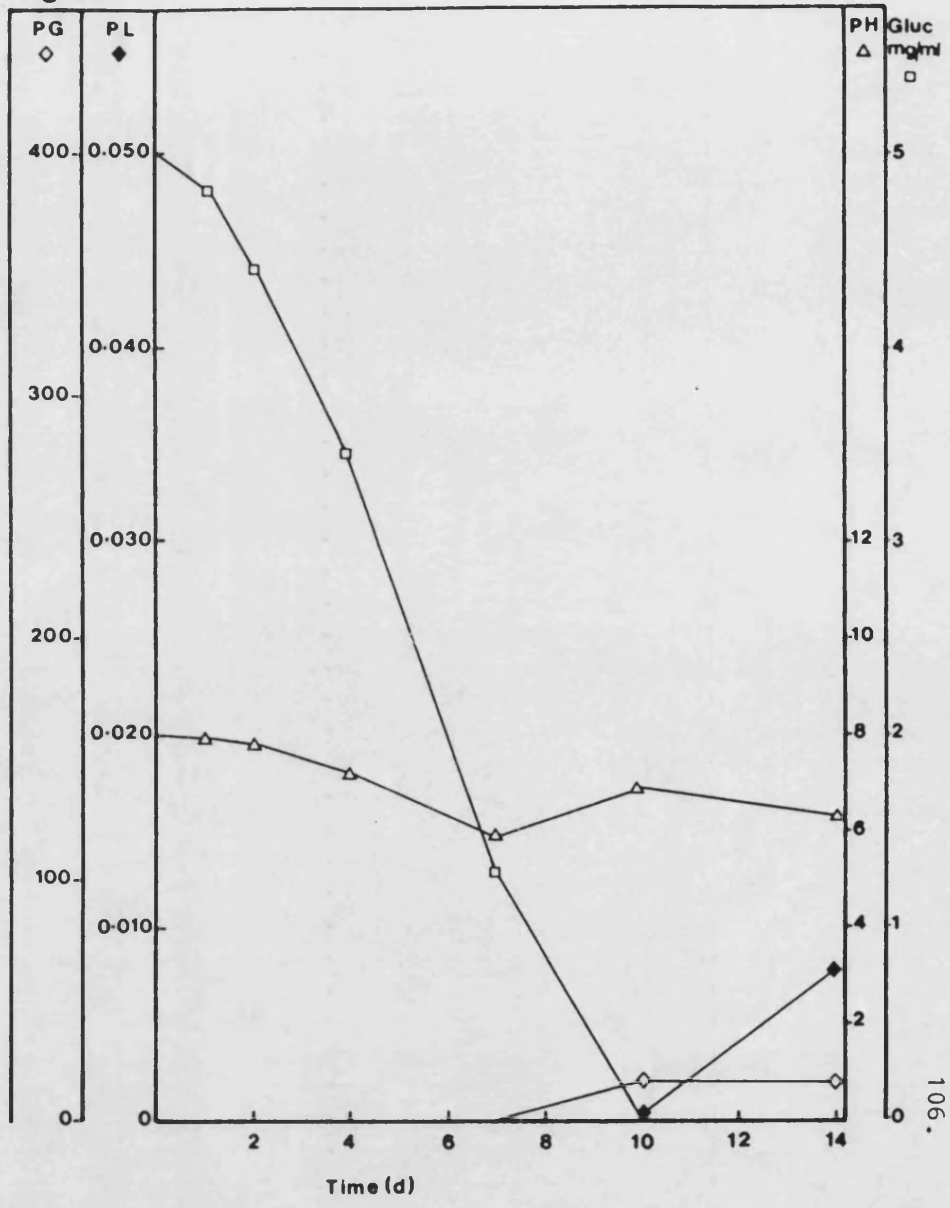
14
Time (d)

Fig 19


PG
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PH
Gluc mg/ml
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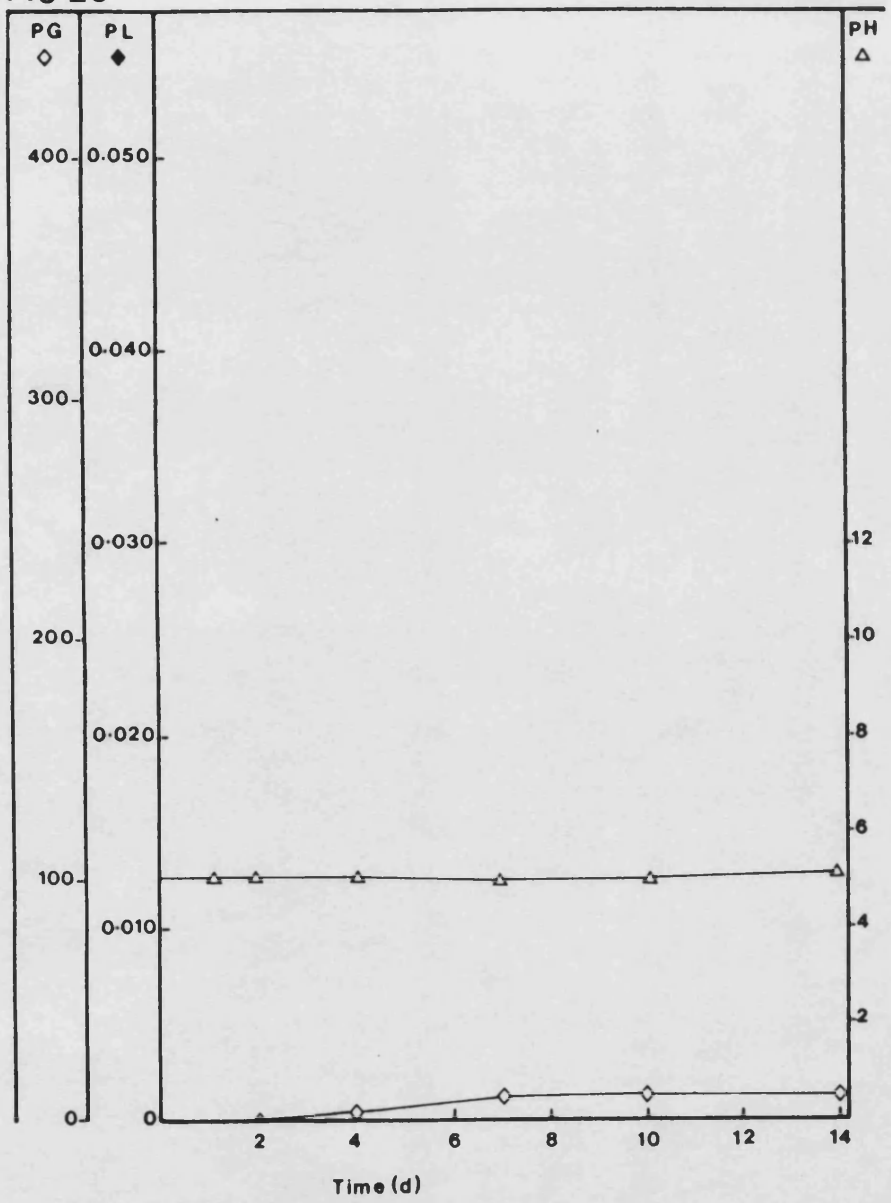
10
8
6
4
2
5
4
3
2
1
0
2
4
6
8
10
12
14
Time (d)

Fig 20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8
6
4
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2
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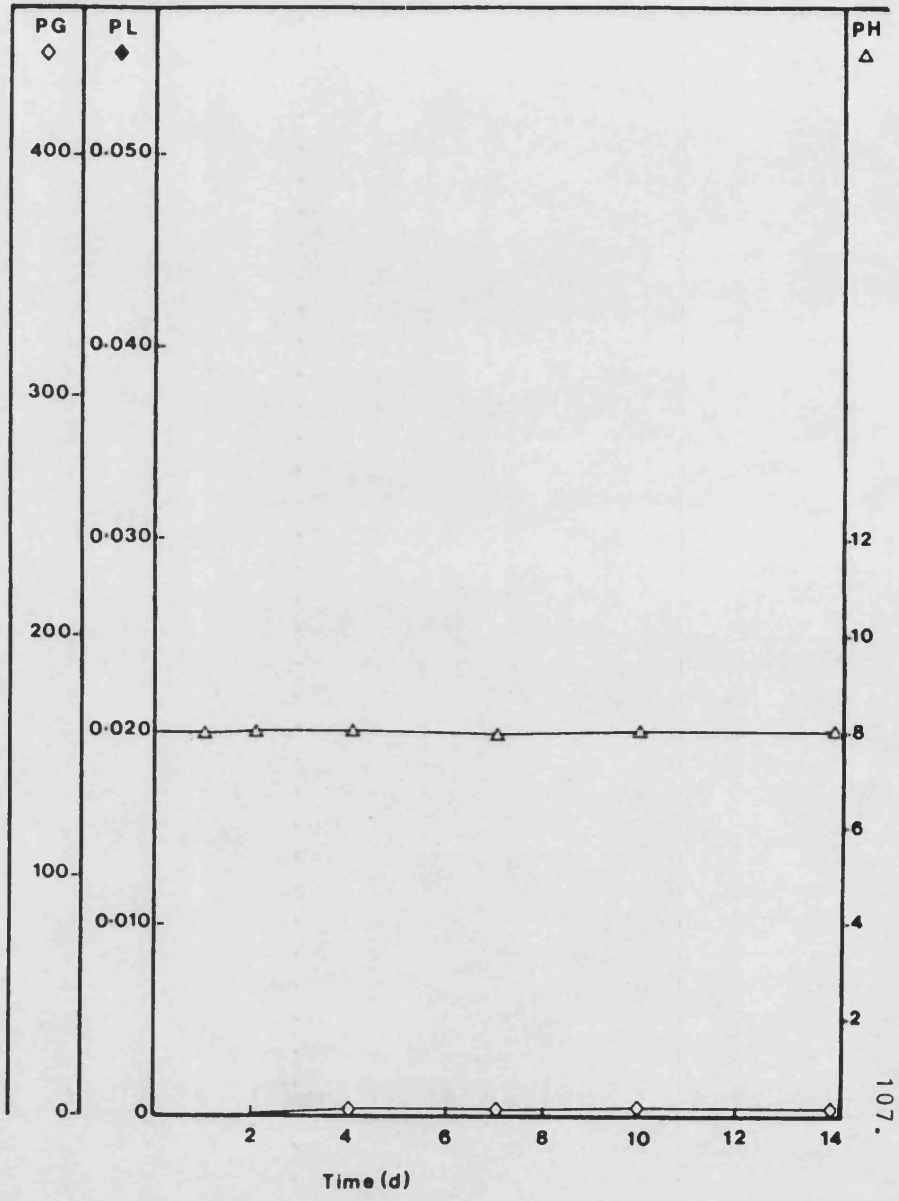
6
8
10
12
14
Time (d)

Fig 21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2
2
4
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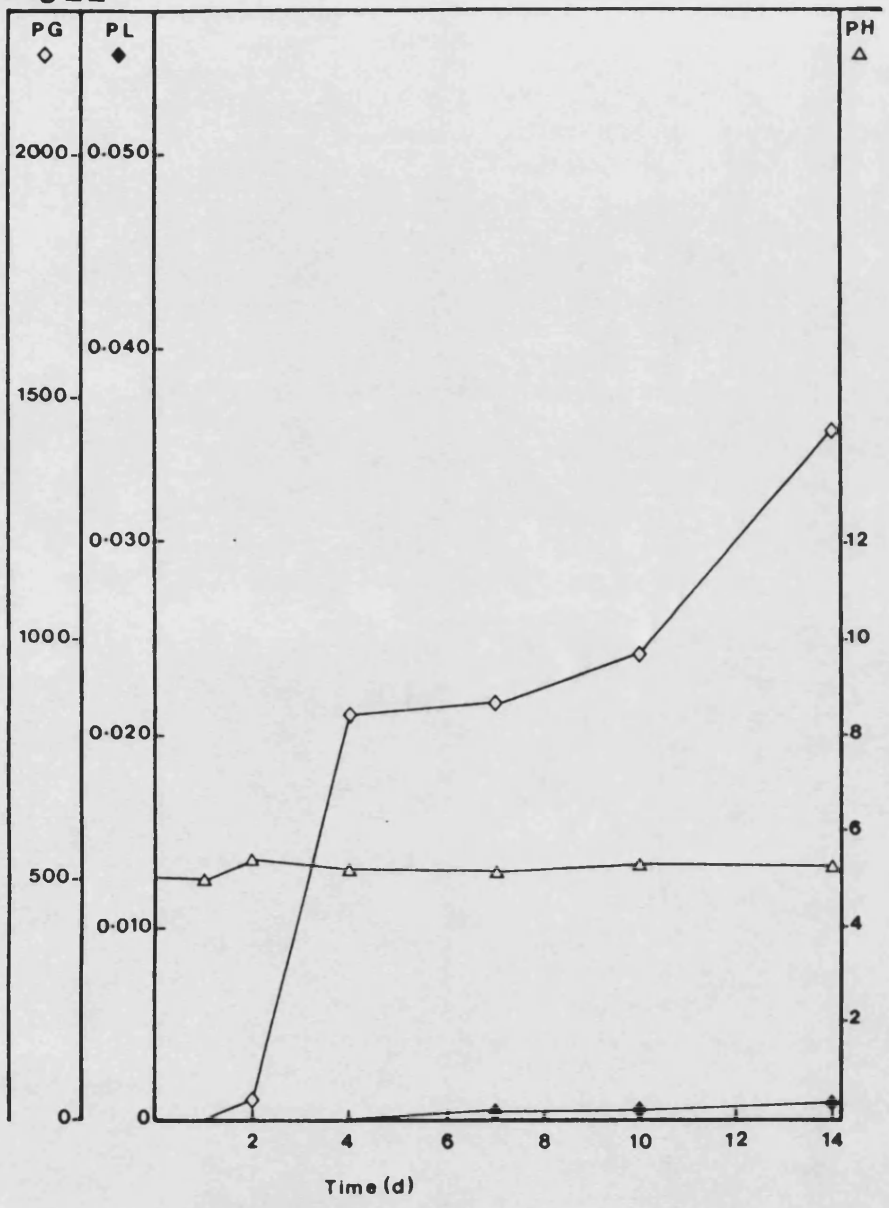
8
10
12
14
Time (d)

Fig 22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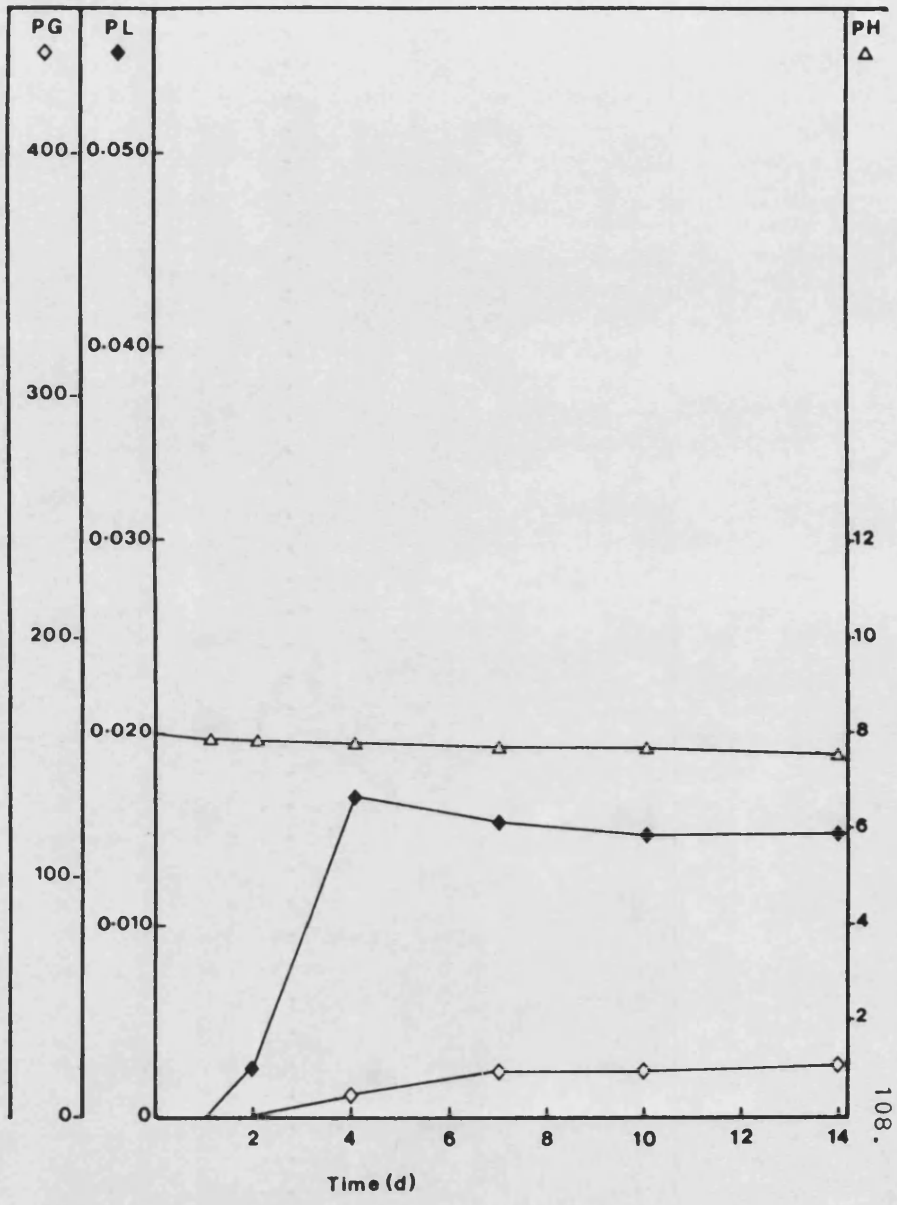
0·010
0
12
10
8
6
4
2
2
4
6
8
10
12
14
Time (d)

Fig 23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8
10
12
14
Time (d)

production was favoured in acidic and alkaline conditions, respectively.

A range of esterases, aminopeptidases, glycosidases, a trypsin and an N-acetyl-β-glucosaminidase were additionally produced on pectin and cell walls by *B. allii* (Appendix 5). A relatively high activity of the latter enzyme was found in the pectin media, which is indicative of autolysis and hyphal wall degradation (R. St. Leger pers. comm.). As many of these enzymes were found in the filtrates of pectin cultures they may be constitutively produced. Less probably, they may have been induced by impurities in this polymer. With the exception of mannosidase and trypsin, a similar range of enzymes was also produced by an isolate of *B. fabae* (Appendix 6) and the VAA +Type (Results and Discussion 3.2.2. In addition to these enzymes VAA and *B. allii* were also shown to possess peroxidase activity. Green clearing zones were evident in rust-red haemoglobin media beneath the developing colonies. As the zones did not extend beyond the colony edge and activity seemed to be restricted to the hyphae the enzyme was presumably hyphal bound.

Neither VAA nor *B. allii* were capable of growing on MES and HEPES, therefore these buffers would not cause CR and may be regarded as biologically inert.

The addition of glucose to the pectic media resulted in CR and induction was delayed, but when the glucose had been metabolised, high levels of PG and PL were synthesised and released (Figs 17 and 18). Basal levels of PG produced on glucose were relatively higher (10 % of induced levels) than those of VAA (3 % of induced levels).

To determine whether PG was produced during the growth phase, dry weights of replicate pectin and pectin/glucose cultures were destructively harvested over time. Levels of GALA residues were also assayed (Figs 24 and 25).

Fig 24. Growth of *B. allii*, mg flask^{-1} (▼), accumulation of GALA, mg ml^{-1} (O) and PG production (◇) in pectin medium (0.5 %; pH 5.0, MES 0.05 M). Mean results of 4 cultures.

Fig 25. Growth (▼), accumulation of GALA mg ml^{-1} (O), glucose (□) mg ml^{-1} and PG production (◇) in pectin (0.5 %) and glucose medium (1 % w/v; pH 5.0, MES 0.05 M). Mean results of 4 cultures.

Fig 24

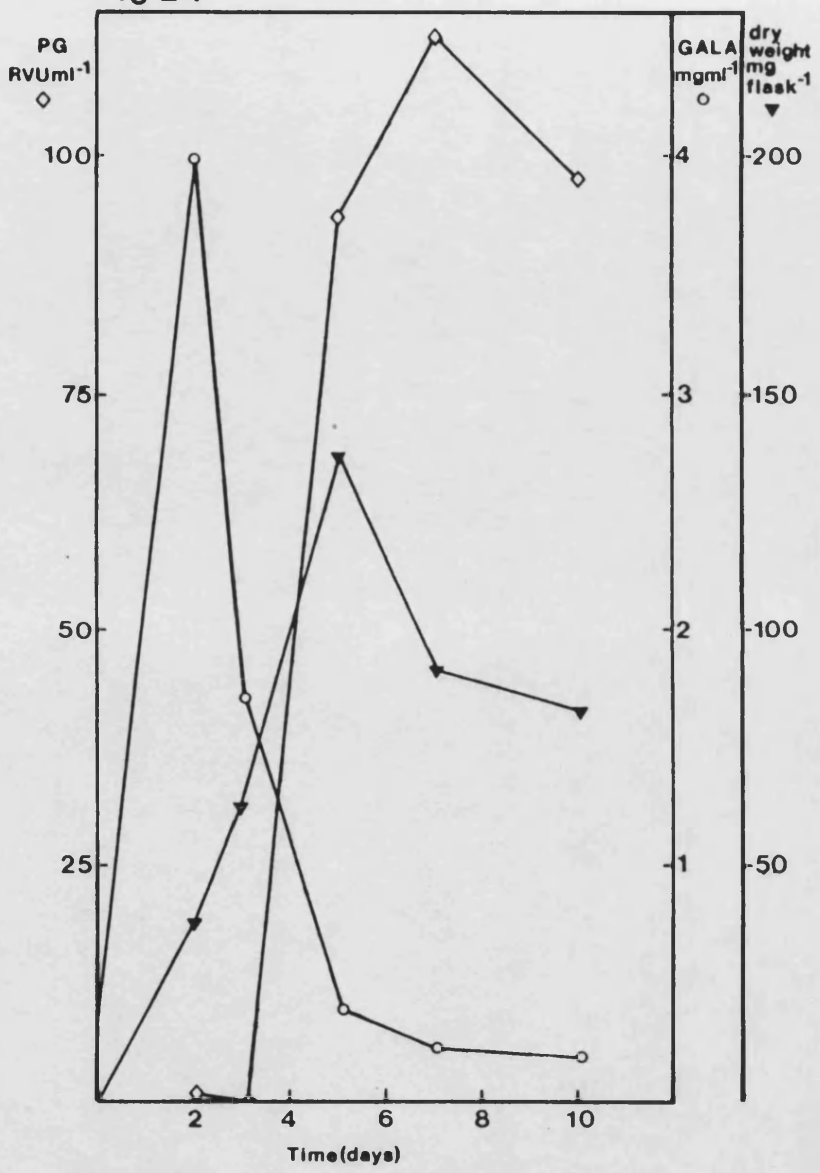
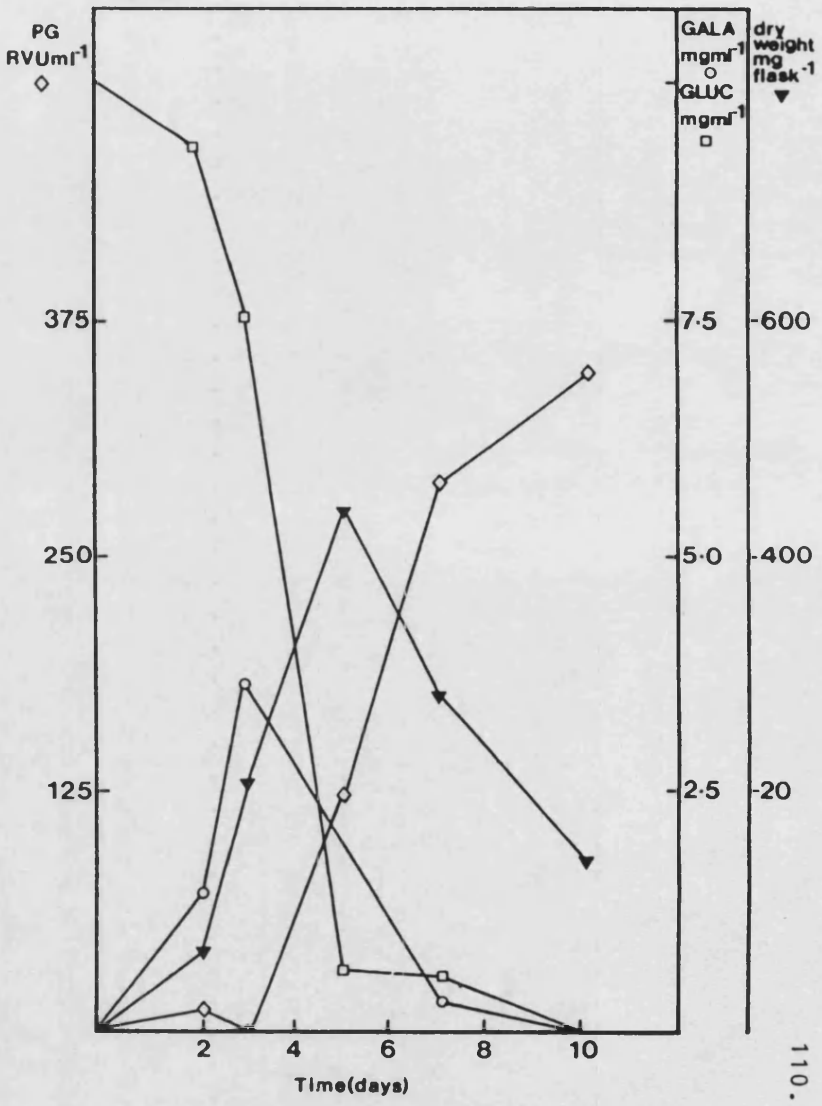

PG
RVUml-1
◇
GALA
mgml-1
○
dry
weight
mg
flask-1
▼
100
75
50
25
4
3
2
1
200
150
100
50
2
4
6
8
10
Time(days)

Fig 25


PG
RVUml-1
◇
GALA
mgml-1
○
GLUC
mgml-1
□
dry
weight
mg
flask-1
▼
375
250
125
7·5
5·0
2·5
600
400
20
2
4
6
8
10
Time(days)

Growth was rapid on pectin and pectin/glucose until 5 d when substrate levels (as determined by glucose oxidase and TBA) had become very low and the cultures had begun to autolyse. Free GALA was not detected in the cultures prior to inoculation but there was an initial accumulation of GALA residues in the first 2 d of growth as the pectin was degraded, although PG levels were still very low at this time. Significant PG activity was not detected on pectin until the levels of GALA residues had fallen to <500 $\mu g\ ml^{-1}$ and CR was presumably relieved. Furthermore, when glucose and GALA levels had fallen in the pectin/glucose media to *c* 200 and 600 $\mu g\ ml^{-1}$ respectively, PG levels began to rise rapidly. *B. allii* was slightly more sensitive to CR than VAA as GALA induced PG synthesis only at concentrations below 500 $\mu g\ ml^{-1}$ in unrestricted cultures.

In conclusion PG production was not directly related to growth but was governed by the levels of GALA in the media.

1.2. Regulation of PG production in restricted *B. allii* cultures.

Following the methods of Pirt (1971) and Cooper & Wood (1975), an attempt was made to determine whether mono-GALA is an inducer of PG, when CR was avoided by supplying the monomer to *B.allii* at slow rates from diffusion capsules. Glucose was similarly used to establish whether PG production was constitutive.

GALA was utilised very slowly by *B. allii* and remained in excess (*c* 350 $\mu g\ ml^{-1}$) even in those cultures fed at the lowest rate of 8 $\mu g\ ml^{-1}\ h^{-1}$ (*B. allii* also grew slowly in unrestricted 100 ml GALA cultures (0.5 % w/v, pH 6.5), attaining a mycelial dry weight of only 30 mg after 7 d). At feed rates of 23 and 43 $\mu g\ ml^{-1}\ h^{-1}$ growth increased marginally and GALA remained at 650 and 1000 $\mu g\ ml^{-1}$ in the cultures after 48 h incubation. PG was found at basal levels in cultures fed

Table 7. Growth and production of PG in *B. allii* cultures supplied with GALA and glucose at constant rates. *a*

Rate of supply of sugar ($\mu g\ ml^{-1}\ h^{-1}$) *b*	sugar conc. at 48 h ($\mu g\ ml^{-1}$)	final dry weight (mg)	PG (RVU) *c*
GALA			
8.0	350	315	84
23.0	650	336	1
43.3	1000	380	1
Glucose			
8.0	180	325	1
23.0	380	382	1

a Results are means from 2 experiments and 2 replicate flasks for each treatment. Initial dry weight was *c* 300 mg.
b GALA and glucose supplied *via* diffusion capsules as described in Materials and Methods 1.3.2.
c Determined viscometrically. PL activity was not detected.

with glucose at 8 and 23 $\mu g\ ml^{-1}$, and, in cultures fed at the higher rates with GALA, thus PG is not produced constitutively. PG was only detected at levels approaching those expected from induced synthesis when GALA was supplied at 8 $\mu g\ ml^{-1}\ h^{-1}$. Therefore PG is induced, at least to some extent, by mono-GALA and synthesis is subject to CR.

1.3. *De novo* synthesis of PG and PL.

The appearance of PG and PL in the presence of pectin could either result from induction and *de novo* synthesis or by post-translational alterations to inactive precursors of the enzymes. To investigate this, cyclohexamide was added to established cultures of VAA and *B. allii* in the presence of pectin which served to induce PG and PL.

Fig 26. Effect of cyclohexamide on PG accumulation in established cultures of *V. albo-atrum*.

VAA was initially cultured in unbuffered glucose media (0.5 % w/v, pH 6.5). 100 ml pectin (1 % w/v; pH 5.0, MES 0.05 M) was added to the established cultures when the glucose concentration had fallen to <0.04 $\mu g\ ml^{-1}$. After 5 h incubation 2 mg cyclohexamide (20 $\mu g\ ml^{-1}$) were added to 4 replicate cultures.
PG was assayed viscometrically; activity is expressed as RVU mg^{-1} mean final mycelial dry weight of 4 cultures.
Final dry weight of cultures; 84.2 ± 3.53 mg (+ cyclohexamide O); 194.5 ± 5.3 mg (- cyclohexamide ●). (SE, n = 4).

Fig 27. Effect of cyclohexamide on PL accumulation in established cultures of *V. albo-atrum*.

VAA was initially cultured in unbuffered glucose media (0.5 % w/v, pH 6.5). 100 ml pectin (1 % w/v; pH 8.0, HEPES 0.05 M) was added to the established cultures when the glucose concentration had fallen to <0.04 $\mu g\ ml^{-1}$. After 5 h incubation 2 mg cyclohexamide (20 $\mu g\ ml^{-1}$) were added to 4 replicate cultures.
PL was assayed by TBA, activity is expressed as $\mu g\ ml^{-1}\ h^{-1}\ mg^{-1}$ mean final mycelial dry weight of 4 cultures.
Final dry weight of cultures; 91.75 ± 4.1 mg (+ cyclohexamide Δ); 221.0 ± 7.2 mg (- cyclohexamide ▲). (SE, n = 4).

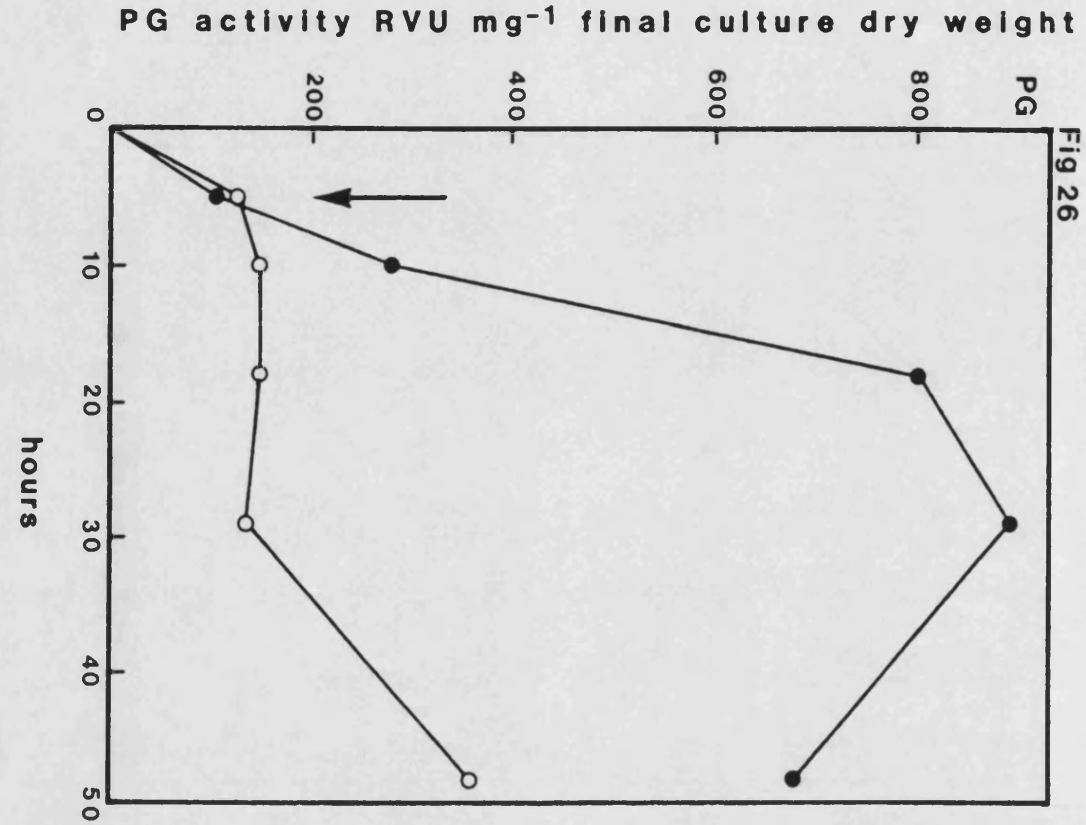

PG activity RVU mg⁻¹ final culture dry weight
PG
0
200
400
600
800
hours
0
10
20
30
40
50

Fig 26

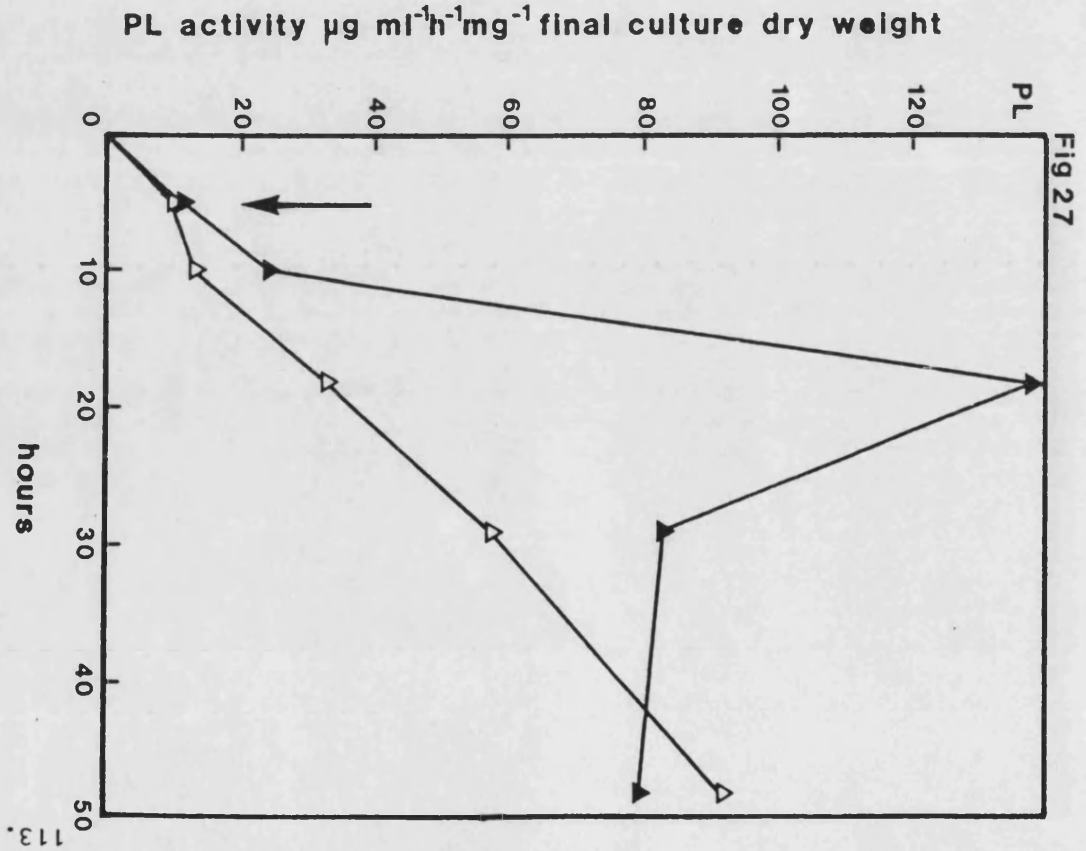

PL activity µg ml⁻¹h⁻¹mg⁻¹ final culture dry weight
PL
0
20
40
60
80
100
120
hours
0
10
20
30
40
50

Fig 27

Fig 28. Effect of cyclohexamide on PG accumulation in established cultures of *B. allii*.

B. allii was initially cultured in unbuffered glucose media (0.5 % w/v, pH 6.5). 100 ml pectin (1 % w/v; pH 5.0, MES 0.05 M) was added to the established cultures when the glucose concentration had fallen to <0.04 μg ml^{-1}. After 5 h incubation 2 mg cyclohexamide (20 μg ml^{-1}) were added to 4 replicate cultures.
PG was assayed viscometrically, activity is expressed as RVU mg^{-1} mean final mycelial dry weight of 4 cultures.
Final dry weight of cultures; 84.2 ± 3.53 mg (+ cyclohexamide O); 194.5 ± 5.3 mg (- cyclohexamide ●). (SE, n = 4).

Fig 29. Effect of cyclohexamide on accumulation of PL in established cultures of *B. allii*.

B. allii was initially cultured in unbuffered glucose media (0.5 % w/v, pH 6.5). 100 ml pectin (1 % w/v; pH 8.0, HEPES 0.05 M) was added to the established cultures when the glucose concentration had fallen to <0.04 μg ml^{-1}. After 5 h incubation 2 mg cyclohexamide (20 μg ml^{-1}) were added to 4 replicate cultures.
PL was assayed by measuring absorbance at 238 nm, activity expressed as μmol ml^{-1} min^{-1} mg^{-1} mean final mycelial dry weight of 4 cultures.
Final dry weight of cultures; 65.7 ± 3.7 mg (+ cyclohexamide Δ); 133.4 ± 7.2 mg (- cyclohexamide ▲). (SE, n = 4).

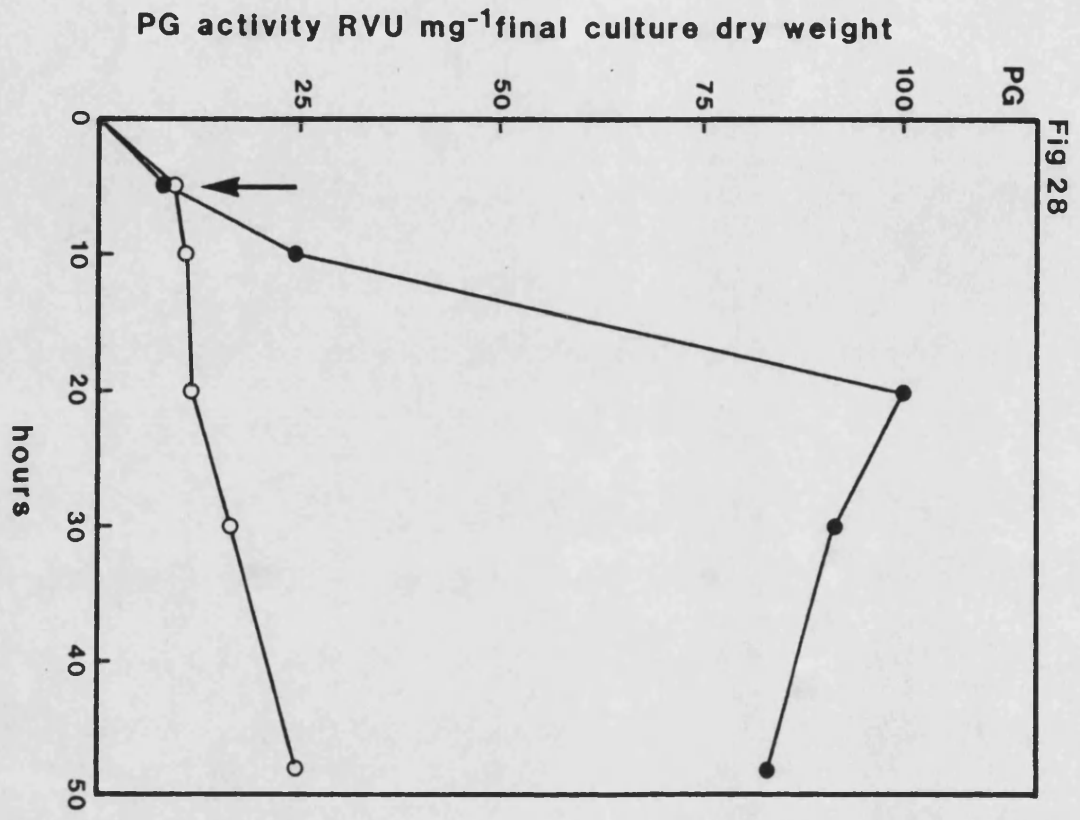

PG activity RVU mg^{-1}final culture dry weight
PG
25
50
75
100
hours
0
10
20
30
40
50

Fig 28

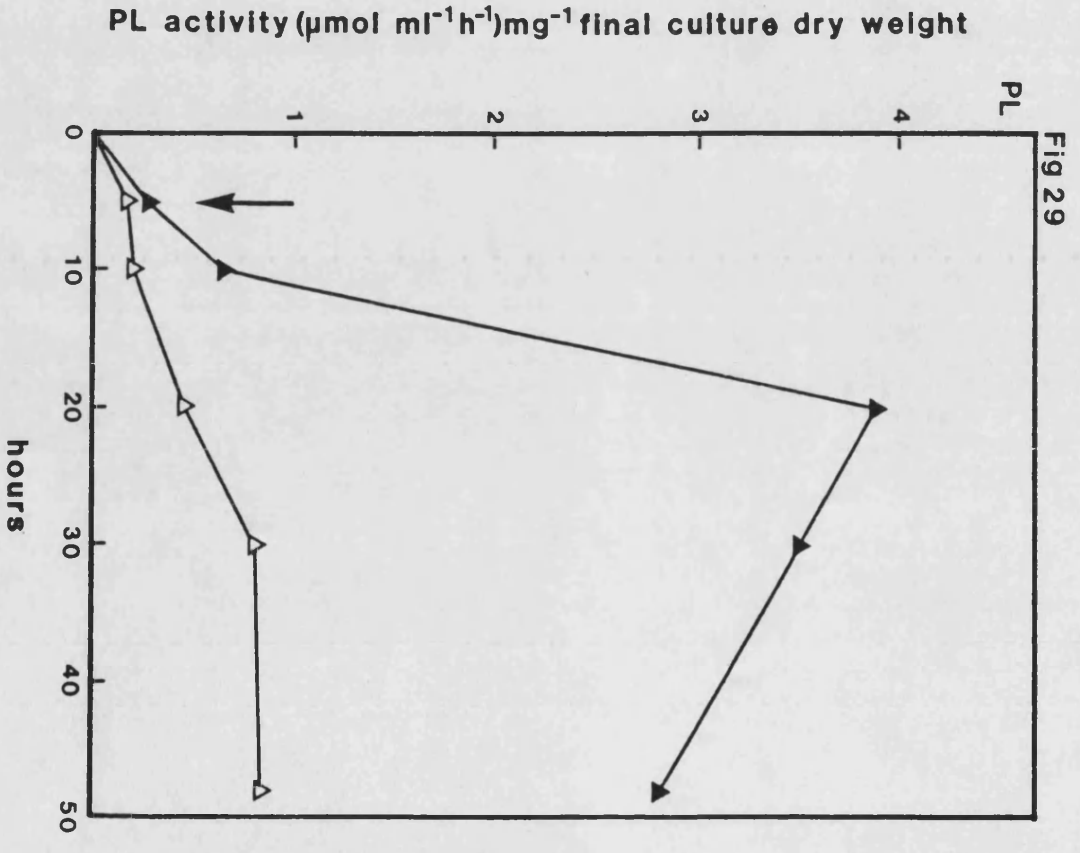

PL activity (μmol ml^{-1}h^{-1})mg^{-1} final culture dry weight
PL
1
2
3
4
hours
0
10
20
30
40
50

Fig 29

1.3.1. *V. albo-atrum.*

VAA was cultured for 3 d on 100 ml unbuffered glucose/salts medium (0.5 % w/v, pH 6.5) until glucose levels were <0.04 μg ml^{-1}. 100 ml of 1 % pectin salts media buffered at either pH 5 (MES, 0.05 M) or pH 8 (HEPES 0.05 M) was added to each culture (final vol 200 ml), to induce PG or PL respectively. The media were gently mixed by shaking and samples were removed for enzyme assay. After 5 h incubation 2 mg cyclohexàmide was added to each flask *via* a millipore filter (0.22 μ) and further samples were removed periodically up to 48 h.

PG accumulation was almost completely inhibited by the addition of cyclohexàmide and only proceeded after 29 h when the fungus recovered from the effects of the inhibitor (Fig 26). Furthermore, the rate of PL accumulation was also reduced by 6.5 fold (Fig 27). Cyclohexàmide is an inhibitor of protein synthesis, therefore accumulation of PG and PL probably involved *de novo* synthesis.

1.3.2. *B. allii.*

Using the procedure outlined in the preceding section cyclohexàmide was added to similar pectin/salts cultures of *B. allii* to determine the effects on PG and PL production.

The accumulation of PG and PL by *B. allii* was also inhibited by cyclohexàmide (Figs 28 and 29), therefore production was also apparently dependent on *de novo* synthesis.

1.4. Production of PG and PL by *B. allii in vivo.*

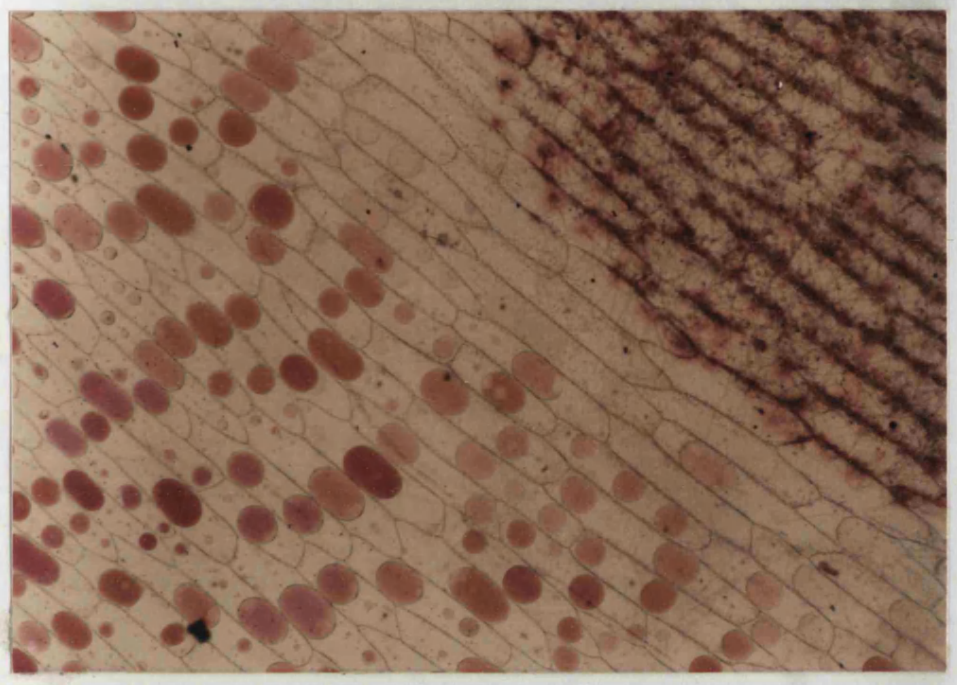
In determining whether *B. allii* PG and/or PL have a rôle in infection and cell killing it was important to find the enzymes in diseased tissue and ideally before or coincidentally with the formation of symptoms.

The inner epidermal surfaces of onion segments were inoculated with 20 µl droplets containing 2 x 10^4 spores from 10 d old YEP plates. Several spore densities were tested in preliminary experiments and this one was found to lead to the formation of a spreading lesion after 3-5 d incubation (see Appendix 7; Stewart & Mansfield, 1985a). As this spore concentation consistently produced spreading lesions on 86 % of inoculated segments, it proved to be ideal for use as a convenient pathogenicity test. 5 mm plugs (*c* 0.12 g and 4 mm thick) were excised from the inoculation sites after 24, 48, 72 h. Tissue was also removed from the lesion edges, upto 2 mm into the surrounding healthy tissue. Extracts of *c* 5 g tissue were made in Na acetate buffer as outlined in Materials and Methods Section 11. The extracts (50 ml) were dialysed and concentrated against polyethylene glycol (MW 20 000) and diluted to 5 ml with d. H_2O.

Epidermal strips, removed from the inoculation sites, were plasmolysed and stained with neutral red to reveal areas of dead, non-plasmolysing cells (Plate 1). Lesion diameters were measured with calipers and fungal development was followed by examining inoculation sites with a light microscope.

Control segments inoculated with autoclaved spores did not show symptoms. 96 ± 2.2 % of the conidia germinated and penetrated the cuticle after 24 h. Neutral red staining showed that by 48 h, a radial zone 2-3 cells in width, of non-staining host cells occurred, indicating cells killed in advance of the colonising fungus. After 72 h the fungus had extensively colonised the inoculation sites and spread into the surrounding tissue. By this time the necrotic lesions were characteristically yellow/brown in colour and slightly depressed. Immersion in the cationic stain ruthenium red showed that hyphal penetration was closely associated with marked wall swelling and dye

Plate 1



Onion bulb epidermis 48 h after inoculation with *B. allii* spores.

Epidermis (5 x 5 mm) was stripped from 2 x 3 cm sections of mid-bulb scales previously inoculated with 20 µl droplets containing 2 x 10^4 spores (Materials and Methods 16.2.). The squares were placed under plasmolysing conditions and stained with neutral red (Materials and Methods 17.3.). Vacuoles of healthy cells stained red; cells beneath the fungal colony and in advance of hyphae were unviable and thus remained unstained.

Table 8. PG and PL activity in lesion extracts from onion bulb scales infected with *B. allii*. (*a*, *c*)

	Lesion Diameter (mm)	Enzyme activity in extract ml^{-1} (*b*)	
		PG (RVU)	PL ($\mu mol\ ml^{-1}\ min^{-1}$)
Control (48 h)	0	0	0
Infected (24 h)	3.5	1.5	0.11
Infected (48 h)	5.0	7.5	0.16
Infected (72 h)	6.0	21.5	0.17

a Inoculated and control tissue was incubated at 18°C.
b PG assayed viscometrically. PL assayed by measuring increase in absorbance at 238 nm.
c Results are means of three separate experiments.

retention whilst the unaffected areas remained comparatively pale. Walls surrounding cells in older lesion areas were swollen even at a distance from penetrating hyphae.

PL was detected in lesion areas after 24 h but levels did not markedly increase beyond 48 h, whereas PG levels continued to increase up to 72 h. Highest PG and PL levels were found within lesions although trace levels of both enzymes (up to 1.5 RVU and 0.010 $\mu mol\ min^{-1}$ in 1 g of tissue) could be detected in tissue removed from 2 mm beyond the area of fungal colonisation, but not in extracts taken from beyond the zone of dead host cells.

Esterase activity was detected on spores and infection hyphae of *B. allii* (Appendix 4) and *B. fabae*, after histochemical treatment. This could have been cutinase activity. Lesion extracts were also tested on API-ZYM strips, which revealed the presence of a range of activities which corresponded to those enzymes produced *in vitro* (Appendix 5). However it is possible that some of these enzymes may have originated from the host. Lesion extracts were also subject to IEF to establish

whether the pectinases produced in onion tissue by *B. allii* were the same isozymes as produced by the pathogen *in vitro*; the results are reported in Results and Discussion 2.1.8. Infection droplets were removed from the epidermal surface after 48 h and centrifuged to remove fungal hyphae. However extracellular PG and PL activity was not found in 1 ml of germination fluid when tested by viscometry and UV absorbance respectively.

1.5. Discussion.

VAA PG and PL were induced on either unmethylated or methylated pectin although their relative activities on these two substrates differed (Cooper & Wood, 1975; Cooper *et al.*, 1978). PME is produced by both VAA and *B. allii* and therefore degradation of pectin could be achieved by PG (Hancock *et al.*, 1964a; Talboys & Busch, 1970). The relative amount of PG and PL induced in pectic media was largely determined by media pH. The appearance of PL in the acidic pectin media coincided with an alkaline drift in pH, whereas PG levels were constrastingly low in NAPP media in which the pH remained *c* 8.0.

PG, which accumulates in acidic pectin media, is relatively unstable in alkaline conditions whereas PL levels are highest in alkaline pectin culture, and is less stable in acidic conditions (see Appendices 7 and 8) Variation in pH stability of PG and PL was indicated by Cooper (1974). However, as three buffering systems were used to produce a pH range of 3-11, buffer effects may have contributed to destabilisation of the enzymes. In an effort to avoid possible buffer effects, Universal buffer was used in the present work.

Although the relative production of PG and PL may be regulated by an unknown pH mediated mechanism, these studies on enzyme stability suggest that differential activity in the acidic and alkaline media result from pH destabilisation.

Although both PG and PL are induced by saturated mono-GALA (Cooper & Wood, 1975), separate metabolic pathways may exist in VAA that lead to induction of PG and PL by saturated or unsaturated GALA. However, transcription of the PG and PL codons may be under the overall control of a single regulatory gene which is duly activated by the products of uronide metabolism. There is a need for a thorough study of assimilation and metabolism in VAA using mono- and oligo- saturated and

unsaturated uronides to determine the exact enzyme mechanisms and metabolites that control induction.

Maximal PL production by VAA occurred 6 d earlier than PG (Figs 6 and 7). The delay in peak PG production may have been a consequence of the initial low media pH (*c* 4). Peak PG production coincided with a pH drift to *c* 5.5 which is almost the optimal pH for this enzyme's stability (Appendix 9.1.). Alternatively, the difference in peak PG and PL production could have resulted from independent induction mechanisms. The predominance of PG in acidic conditions coincided with the, accumulation of saturated breakdown products. In contrast, when PL was the predominant enzyme, unsaturated oligo-galacturonic acids were mainly produced. The relative assimilation and metabolism of uronides could be the rate limiting step in determining the timing of pectinase induction, as is the case with *E. chrysanthemi*; saturated di-GALA is cleaved more slowly by intracellular OGL than unsaturated di-GALA of Ech (Collmer & Bateman, 1981; Collmer *et al.*, 1982b). Thus in the Ech system PGL is induced more rapidly in the presence of di-UGALA and the period of CR is extended when di-GALA is provided because metabolism of the saturated uronide is considerably slower. It is plausible that VAA is likewise less able to utilise saturated products and that CR is imposed for longer than in the presence of similar levels of UGALA oligomers.

The appearance of PG and PL on acidic and alkaline pectic media by *B. allii* was very similar to VAA. Synthesis was also induced to some extent by GALA although growth on the monomer was poor which suggests that other saturated or unsaturated oligo-galacturonic acids may be more potent inducers. Metabolism of mono-GALA may be unimportant for these fungi, both in culture and during pathogenesis. Thus, an OGL$^-$

mutant of Ech remained pathogenic despite an inability to utilise various uronides.

Induction was prone to CR if GALA was in excess, both in restricted and unrestricted cultures (Figs 24 and 25; Table 7). *B. allii* utilised GALA in unrestricted cultures (0.5 % w/v) but attained only *c* 35 mg mycelial dry weight after 7 d with no production of PG and PL. PG and PL were detected in very low amounts on glucose and CMC, signifying basal synthesis (Figs 18-21). High levels of pectinases were produced by *B. allii* only in the presence of rhamnogalacturonan when provided separately or as a component of onion cell walls (Figs 14, 15, 22 and 23); uronic acid and rhamnose constitute almost 30 % of total wall sugar (Mankarios, Hall, Jarvis, Threlfall & Friend, 1980). The rapid production of PG and PL on cell walls emphasises the amenability of the pectic component to degradation (Mankarios & Friend, 1980). These workers found that cell wall degradation in culture occurred in two stages, involving an initial release of rhamnose, galactose and arabinose, followed by a second group consisting of mannose, xylose, fucose and glucose. The former sugars are associated with pectin degradation whereas the latter are characteristic of hemicellulose and cellulose degradation (Selvendran, 1985); the release of each group coincided with the relative activities of the relevant enzymes in the media at the time of sampling (Mankarios & Friend, 1980). The API-ZYM strips indicated that *B. allii* produces a wide range of glycosidases on onion cell walls *in vitro*, which could readily release many of the sugars detected in culture filtrates by Mankarios & Friend (1980). *B. allii* produced α and β-galactosidase, α and B-glucosidase and α-mannosidase, but no α-fucosidase was detected although fucose is present in slightly higher amounts than mannose in onion cell walls (Mankarios, Jones, Jarvis, Threlfall & Friend, 1979; Mankarios *et al.*,

1980). They found the two galactosidases to be the major hemicellulases, which is interesting as galactose contributes 28.4 % of the pectin fraction (42.4 % of total wall carbohydrate) (Mankarios *et al.*, 1980).

PG and PL production by *B. allii* and VAA involves *de novo* synthesis of protein as accumulation of pectinases was inhibited by cyclohexàmide (Figs 26-29). There are few examples in the literature where workers have tested for *de novo* synthesis of induced polysaccharidases. However, recent work with *M. anisopliae* suggests that N-acetylglucosaminidase, chitinase and protease are also all produced by *de novo* protein synthesis (St. Leger, 1985).

Accumulation of PG and PL eventually occurred when the two plant pathogens had recovered from the effects of the inhibitor, possibly following its degradation. The cyclohexàmide presumably prevented the translation of the PG and PL mRNA which would have otherwise been synthesised on induction by galacturonides. Alternatively, the pectinases were present intracellularly in an inactive form, and the cyclohexàmide inhibited the synthesis of other enzymes and proteins involved in modification of pro-enzymes, prior to excretion; such components would presumably be the products of Out genes (Andro *et al.*, 1984). It may be possible to determine whether the enzymes are actively secreted by employing low concentrations of a metabolic inhibitor, eg, sodium azide.

Total PG activity in infected onion tissue was lower than that detected in extracts from lesions on *Vicia faba* leaves 24 and 72 h after inoculation with *B. fabae* (Balasubramani *et al.*, 1971; Table 8). This difference might be explained by the greater aggressiveness of *B. fabae*, which causes complete tissue disintegration in lesion areas in 72 h. *B. allii* colonises the considerably thicker onion bulb scales at a relatively slower rate (Stewart & Mansfield, 1985) and tissue degradation is less severe.

Cell death was associated with swollen cell walls which stained deeply with ruthenium red. This was also observed by Stewart (1983) under similar conditions and is indicative of pectic degradation. A ring of dead cells typically surrounded the colonising mycelium of *B. allii* (as indicated by their failure to retain neutral red; Plate 1; Stewart, 1983). Cell killing in advance of the fungus could be achieved by the release of a diffusable *endo*-PG. The ability of this enzyme(s) to damage host cells was subsequently shown (Results and Discussion 2.1.9.). Low levels of PG were isolated from within lesions and from around the lesion edges, which further indicates that this enzyme is responsible for causing host damage. It is notable that other workers have found that tissue damage can result from incubation from very low levels of pectinases (eg Keon, 1985). Similarly, on exposure to PG (at higher levels than recovered from infected tissue), onion tissue rapidly lost K^+ ions and viability (Results and Discussion 2.1.9.). It is however imprudent to make direct comparisons between the levels of activity and the effect that pectinases have *in vitro* and *in vivo*, considering the differences in the conditions and the amount of enzyme and tissue involved in each case. Detection of PG *in vivo* and its ability to kill cells *in vitro* is though strong circumstantial evidence for a rôle in pathogenesis.

Levels of PG detected by Hancock *et al.* (1964b) in 10 day old infected detached and intact onion leaves were *c* 10 x higher than the levels found in 3 d old bulb tissue in this study. Hancock *et al.* (1964b) also reported the presence of an exo-polygalacturonase that was induced on onion leaves. Mankarios & Friend (1980) reported a trace of PL activity in the culture filtrate of *B.allii* grown on host cell walls, but no previous workers have shown any other *Botrytis* species to produce PL *in vivo* or *in vitro*.

Comparison of enzyme activities directly with those of other workers must remain tentative as there are many differences in the host tissue, inoculation techniques and extraction methods between laboratories. PG was not found in inoculation droplets on onion epidermis either in this study or by Stewart (1983). Although production was not determined *in vivo*, Kritzman *et al.* (1980) reported constitutive production of PG in inoculation droplets incubated on water agar. However, this was probably basal production as these workers further reported that activities tended to be significantly higher when the media was supplemented with pectin, when the activity presumably resulted from induced synthesis. Conidia of *B. cinerea* contain a constitutive PG which is released on wetting (Verhoeff & Liem, 1978).

Low PG levels in lesion extracts may reflect the vulnerability of the enzyme to the extraction procedures. However, the simple extraction mixture used in this study proved optimal, and the addition of anti-oxidants and phenol adsorbents failed to increase recovery. Detection was particularly difficult in the crucial early stages of infection. Gold labelled anti-bodies raised against the purified enzyme, could provide further evidence for the presence of this enzyme *in vivo*. This technique proved the presence of hyphal-bound PG in bean tissue infected with *C. lindemuthianum* although no PG activity had been previously detected by conventional extraction (O'Connell *et al*, 1986).

PL was produced by *B. allii in vivo* at high levels relative to PG compared to *in vitro*. The former enzyme may be more stable in onion tissue and less prone to the effects of inhibitors which may be present in the tissue. In view of the inability of this *exo*-acting lyase to kill host cells (Results and Discussion 2.1.9.), PL may have an important rôle in nutrition of *B. allii* or in regulation of PG synthesis.

Recovery of low levels of *endo*-PG in the tissue may imply that; (1) low levels are produced *in vivo* by *B. allii*; PG may not be induced in onion tissue after 3 d incubation and the detectable enzyme reflects basal synthesis; (2) activity is lost during extraction; (3) the presence of PG and PL in host tissue at the time of cell death and parasitism is circumstantial and that other pathogen produced factors, eg, some of the other CWDE detected *in vivo* and *in vitro* (Appendix 5) are involved in host colonisation.

It is, however, unlikely that non-pectolytic CWDE are involved in cell killing as hemicellulases do not cause maceration or leakage (Bateman & Basham, 1976).

The esterase activity detected on the surfaces of spores and infection hyphae of *B. allii* and *B. fabae* may be of importance during penetration of the cuticle. Such activity may belong to a cutinase. Although cutinase activity was not directly determined, by using cutin as the substrate (Shishyama *et al.*, 1970a and b), the API-ZYM strips did provide some evidence of the presence of a long-chain fatty acid-esterase. Furthermore a cutinase has been isolated from cutin cultures of *B. cinerea* and from infected tissue (Shishyama *et al.*, 1970b). This and other cutinases have been found to be essential in penetration and are thus of great importance during initial infection (Kolattukudy & Köller, 1983). It would be of interest to determine the importance of cutinase in onion and bean infection by *B. allii* and *B. fabae* respectively.

It is possible that non-enzymic phytotoxic factors are responsible for causing host cell damage. Five fractions of decreasing molecular weight, isolated by Stewart (1983) from culture and infected host tissue, were considered to be the phytotoxic factors responsible for the killing of host cells by *B. fabae* and *B. squamosa*. However the three

unidentified heat stable non-enzymic low molecular weight compounds were only mildly phytotoxic and were considered of relatively little importance. Cell killing *in vitro* was mainly correlated with the presence of PG because toxicity of culture fluids increased on addition of pectin or host cell walls to the minimal media (Stewart, 1983). Presumably the presence of these carbon sources resulted in the induction of PG synthesis. In contrast, cultures of *B. allii*, *B. cinerea*, *B. fabae* and *B. squamosa* which contained glucose remained non-toxic and coincidentally produced very little PG (Stewart, 1983). These results further suggest that polygalacturonase is the factor responsible for toxicity of extracellular fluids. Moreover, the presence of *endo*-PG in tissue infected with *Botrytis* species may explain the characteristic wall swelling and cellular disruption observed by TEM (McKeen, 1974; Stewart & Mansfield, 1985a).

H_2O_2, produced by peroxidase (or glucose oxidase) activity, has been shown to be important in wood degradation by fungi (Koenigs, 1972). For this reason the production of H_2O_2 by *B. allii* was monitored on blood agar plates (Koenigs, 1972). However, activity was limited to the colony surfaces, which suggested that *in vivo* peroxide may not be involved in wall degradation and/or killing of host cells in advance of colonisation. Free H_2O_2 has been claimed to cause chlorosis in cotton infected with *V. dahliae* (Mussell & Strand, 1977). However, production of H_2O_2 by VAA would presumably be of little importance *in vivo* as it was apparently limited to the surfaces of the hyphae and did not diffuse beyond the colony edges on blood-agar. H_2O_2 could only be responsible for causing chlorosis if it was extracellular because the symptoms occur remotely from the fungus, which is confined to the vascular system. Furthermore, pathogenesis has only been associated with the formation of free H_2O_2 by 11 species of white rot fungi (Koenigs, 1972).

2. Properties of the polygalacturonases and pectin lyases produced by *B. allii*, *V. albo-atrum* and *V. dahliae*.

The properties of PG and PL were investigated to ascertain optimum conditions for assay, purification, design of mutant-selection media and to gain knowledge of the mechanisms of action on substrates and host tissue. This was mainly done with partially purified enzymes. As many of the properties of VAA PG and PL have already been determined (eg Cooper *et al.*, 1978), most of the results concern those of *B. allii*.

2.1. *B. allii*.

2.1.1. Substrate specificity and the effect of pH.

2.1.1.1. Polygalacturonase.

PG activity was assayed viscometrically with NAPP (1 % w/v) and pectin (1 % w/v) buffered in Britton and Robinson Universal buffer at a pH range of 3-12. Crude dialysed PG from pectin/salts cultures (pH 5) and the major isozyme of PG (pI 5.4) prepared by IEF fractionation (Fig 36) were assayed against both substrates to determine the pH optimum and substrate specificity.

PG was most active between pH 5 and 7. The pH optimum profile of the major PG isozyme (pI 5.4) is representative of that of the entire PG compliment in crude culture fluids as relative activites of both differed by no more than 2 % (Figs 30 and 31). B. allii PG was more active against NAPP, but was capable of degrading the pectin to some extent because the polymer is incompletely methylated, (75-85 %). The pH optimum and substrate specificity are typical of most PG's (Cooper, 1983). The stability of PG similarly ranged between pH 6 and 7 (Appendix 8).

Fig 30. Effect of pH (3-12) on activity of *B. allii* polygalacturonase.

PG activity in crude dialysed pectin/salts culture (pH 5.0, MES, 0.05 M) filtrate was assayed viscometrically in NAPP (O) or pectin (●) (1 % w/v) at a range of pH (3-10) in Universal buffer.

Maximum PG activity: 110 RVU.

Fig 31. Effect of pH (3-12) on activity of *B. allii* polygalacturonase isozyme (pI 5.4).

Enzyme activity of the major PG isozyme, resolved by broad range IEF (pH 3-10; see Fig 36) was assayed viscometrically in NAPP (O) or pectin (●) (1 % w/v) at a range of pH (3-10) in Universal buffer.

Maximum PG activity: 120 RVU.

Fig 30

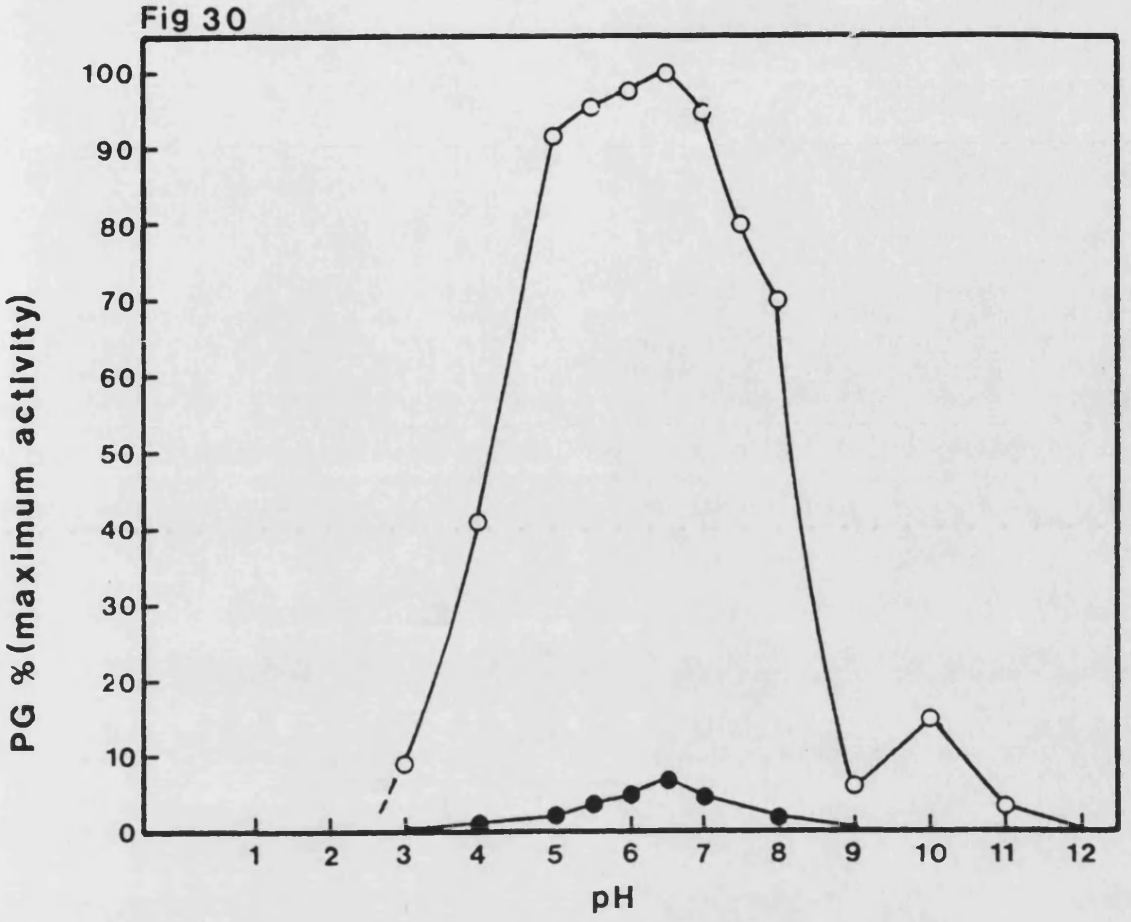

PG %(maximum activity)
100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
7
8
9
10
11
12
pH

Fig 31

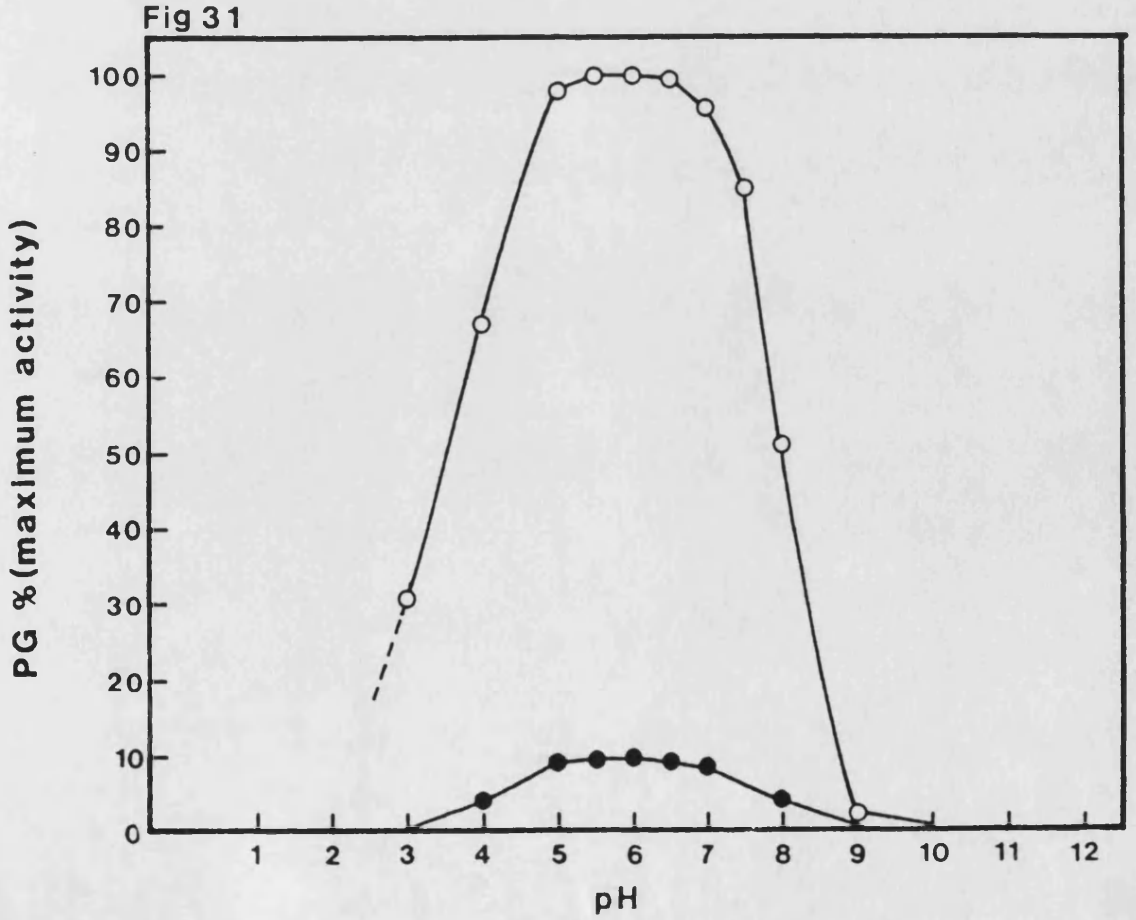

PG %(maximum activity)
100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
7
8
9
10
11
12
pH

Fig 32. Effect of pH (3-11) on activity of *B. allii* pectin lyase.

Enzyme activity of PL (O) in dialysed crude pectin/salts culture (pH 5.0, MES 0.05 M) filtrate was assayed against NAPP and pectin (0.5 % w/v) at a range of pH in Universal buffer. PL activity was determined by TBA because Universal buffer interfered with the UV absorption assay.

Maximum PL activity: 120 $\mu g\ ml^{-1}\ h^{-1}$.

Fig 33. Effect of pH (7-9) on activity of *B. allii* pectin lyase.

Enzyme activity of PL (O) purified by broad range IEF (pH 3-10; Fig 36) was assayed against NAPP and pectin (0.1 % w/v) at a range of pH (7-11) in Tris (0.05 M). PL activity was determined by UV absorption at 238 nm.

Maximum PL activity: 0.020 $\mu mol\ ml^{-1}\ min^{-1}$.

Fig 32

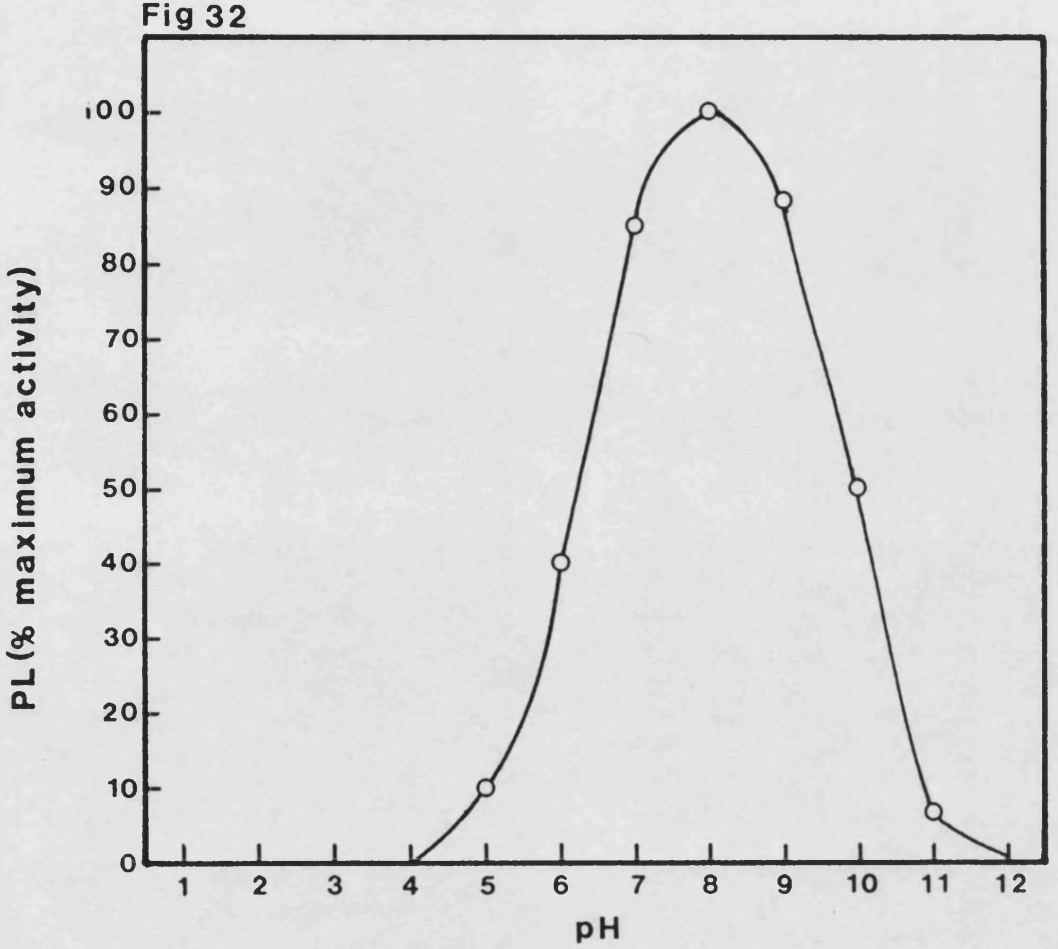
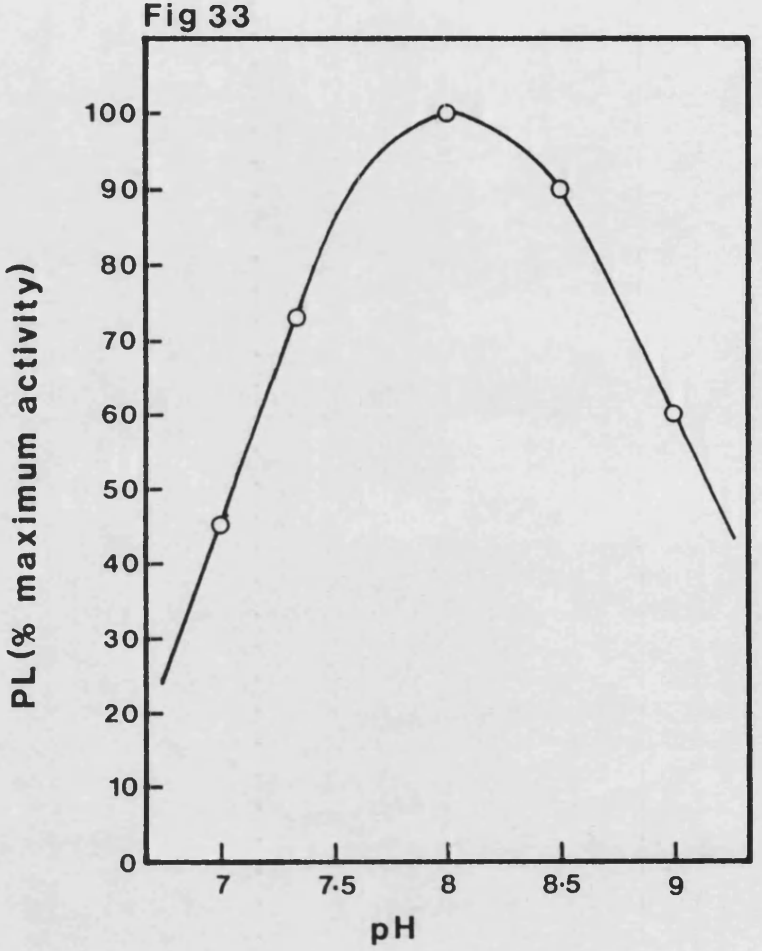

PL (% maximum activity)
100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
7
8
9
10
11
12
pH

Fig 33


PL (% maximum activity)
100
90
80
70
60
50
40
30
20
10
0
7
7·5
8
8·5
9
pH

2.1.1.2. Pectin lyase.

PL of *B. allii* was specific for pectin (Figs 32 and 33); activity on NAPP was not detected by either TBA or the UV absorption assay. *B. allii* produces only one PL enzyme (pI 7.6). The pH optimum for PL activity was 8.0 from either crude dialysed pectin/salts culture filtrate (pH 8.0) or from IEF fractionation (Figs 32 and 33).

After the approximate pH optimum for PL activity had been obtained (Fig 32), a more accurate determination was made by the UV absorption assay with 0.1 % pectin buffered from pH 7-9 (Tris, 0.05 M) (Fig 33). This confirmed that optimal PL activity on pectin occurs at pH 8.0, which is typical of most fungal lyases (Cooper, 1983). Similarly PL was most stable at pH values that were close to its pH optimum (Appendix 9).

2.1.2. The effect of calcium and potassium ions on PG and PL activity.

Fungal pectin lyases often have a complete or at least partial requirement for the presence of Ca^{2+} ions as cofactors during substrate degradation. In contrast, PG activity is often inhibited by Ca^{2+} ions.

2.1.2.1. Polygalacturonase.

Ca^{2+} ions, even at very low concentrations caused rapid gelation of 1 % NAPP and as it was not possible to assay PG viscometrically, activity was determined by TBA.

Increasing levels of $CaCl_2$, KCl and KH_2PO_4 reduced PG activity from dialysed culture filtrate (Fig 34). Ca^{2+} ions were more inhibitive to PG activity than K^+ ions at concentrations ≥ 0.001 M. Ca^{2+} was a more effective inhibitor perhaps because it has the ability not only to inhibit enzyme-substrate bonding but also to form bridges between the polymer chains which render portions unavailable to attack by PG. When K phosphate buffer was used in place of KCl inhibition was not as

Fig 34. Effect of increased concentrations of calcium and potassium ions on *B. allii* PG activity.

PG was assayed by measuring the release of GALA from NAPP (0.1 % w/v, pH 5.0) after 1 h incubation at 25°C by TBA. The reaction mixture comprised 0.5 ml dialysed crude pectin/salts culture filtrate, 0.5 ml NAPP (0. 1 % w/v; pH 5.0, MES 0.4 M) and 0.5 ml salt solution ($CaCl_2$ O, KCl ●, KH_2PO_4/K_2HPO_4, pH 6.0 Δ), at a range of 0.0001-0.1 M. All reagents were dissolved in deionised water.

Maximum PG activity: 180 $\mu g\ ml^{-1}\ h^{-1}$.

Results are representative of three separate experiments.

Fig 35. Effect of increased concentrations of calcium and potassium ions on PL activity.

PL was assayed by measuring the release of UGALA from pectin (0.1 % w/v, pH 8.0) by UV absorption at 238 nm. The reaction mixture consisted of 0.45 ml pectin (0.2 % w/v; pH 8.0, HEPES 0.1 M), 0.45 ml salt solution ($CaCl_2$ O, KCl ●, KH_2PO_4/K_2HPO_4, pH 6.0 Δ), at a range of 0.0001-0.1 M and 0.1 ml enzyme solution. All reagents were dissolved in deionised water.

Maximum PL activity: 0.05 μmol ml-1 min^{-1}.

Results are representative of three separate experiments.

Fig 34

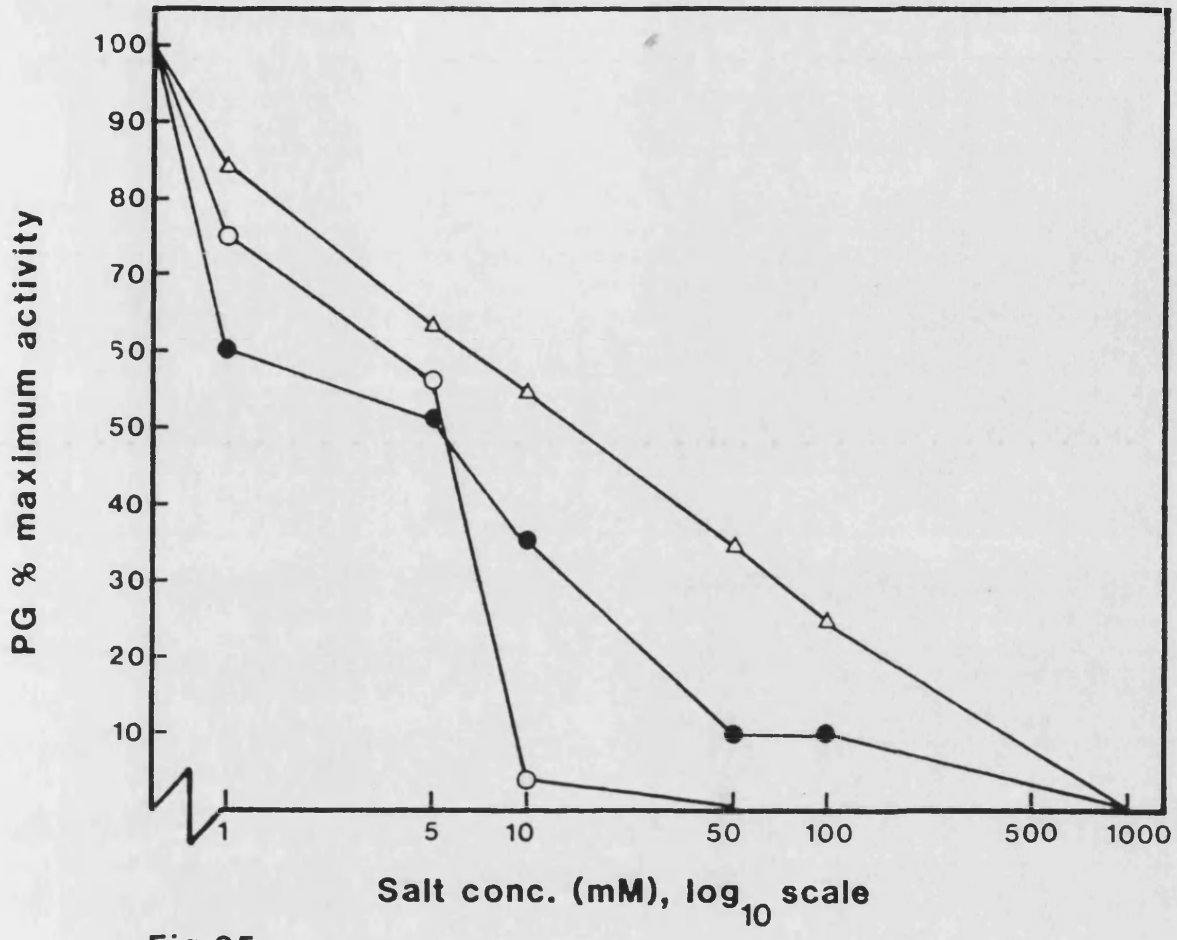
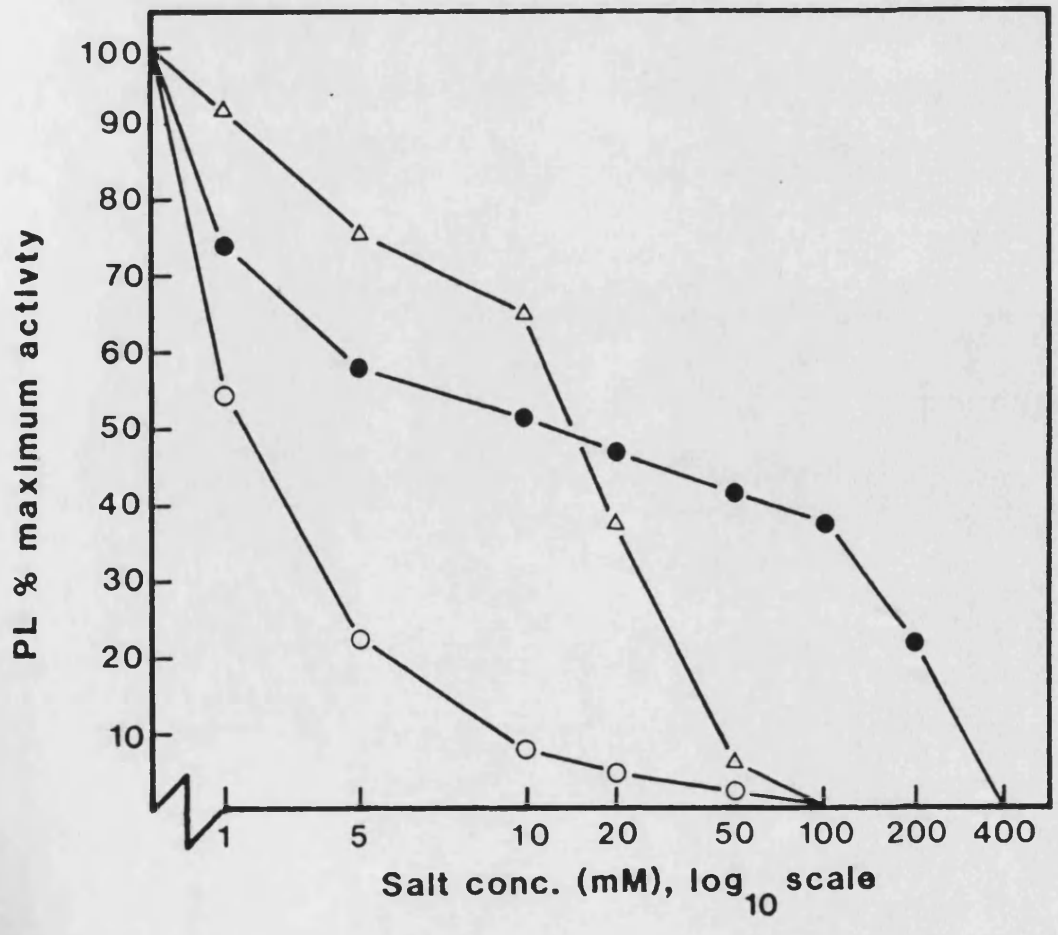

PG % maximum activity
100
90
80
70
60
50
40
30
20
10
1
5
10
50
100
500
1000
Salt conc. (mM), log10 scale

Fig 35


PL % maximum activity
100
90
80
70
60
50
40
30
20
10
1
5
10
20
50
100
200
400
Salt conc. (mM), log10 scale

pronounced. At pH 6.0 the predominant salt in K phosphate buffer is KH_2PO_4. The K^+ concentration would be approximately equal to that in KCl at the same molarity, therefore the Cl^- ions may also inhibit PG.

2.1.2.2. Pectin lyase.

PL activity was determined by UV absorption against 0.1 % pectin containing $CaCl_2$ (0.001-0.1 M). In contrast to most pectin lyases, the *B. allii* PL degraded pectin in the absence of Ca^{2+} ions. In addition, Ca^{2+} ions even at low levels (0.001 M), caused a significant decrease in PL activity (Fig 35). KCl (0.001-0.4 M) was used in place of $CaCl_2$ to determine whether the inhibition of PL by Ca^{2+} was a specific or a non-specific effect. K^+ ions inhibited PL activity 1.5 - 4 fold less than it did PG, at equivalent molarity. K phosphate (pH 8.0, 0.001 - 0.1 M) was also added to the reaction mixture and proved more inhibitive towards PL than KCl; presumably phosphate ions are more inhibitive than Cl^- ions at equivalent molarities.

2.1.3. Enzyme multiplicity as determined by isoelectric focusing.

Flat bed IEF was used to determine the number of PG isozymes produced by *B. allii* and other Botrytis species. Column IEF was used routinely to obtain semi-pure preparations of *B. allii* PG and PL.

2.1.3.1. Flat bed isoelectric focusing.

2.1.3.1.1. IEF profiles of five *B. allii* isolates.

As part of an initial screen for a suitable +Type pathogenic strain of *B. allii* 5 isolates were grown for 7 d on pectin/salts media (pH 5.0). PG and PL activities were determined in the culture filtrates (Table 9), which were duly concentrated by ammonium sulphate precipitation,

Fig 36. Broad range isoelectric focusing (pH 3-10) of polygalacturonase and pectin lyase of *B. allii*. PG (O), PL (●), protein (—), pH (--).

Concentrated pectin/salts culture filtrate (pH 5.0, MES 0.05 M) was fractionated on an LKB 8101 IEF column (110 ml) in a pH gradient 3-10.

Total protein applied to column: 3.5 mg.

Total PG activity applied to column: 20 300

Total PL activity applied to column: 8.67 μmol min^{-1}.

Fraction size: 3.75 ml.

Maximum PG activity (fraction 10): 800 RVU ml^{-1}.

Maximum PL activity (fraction 18): 0.79 μmmol ml^{-1} min^{-1} ml^{-1}.

Results are representative of three separate fractionation experiments.

Fig 36

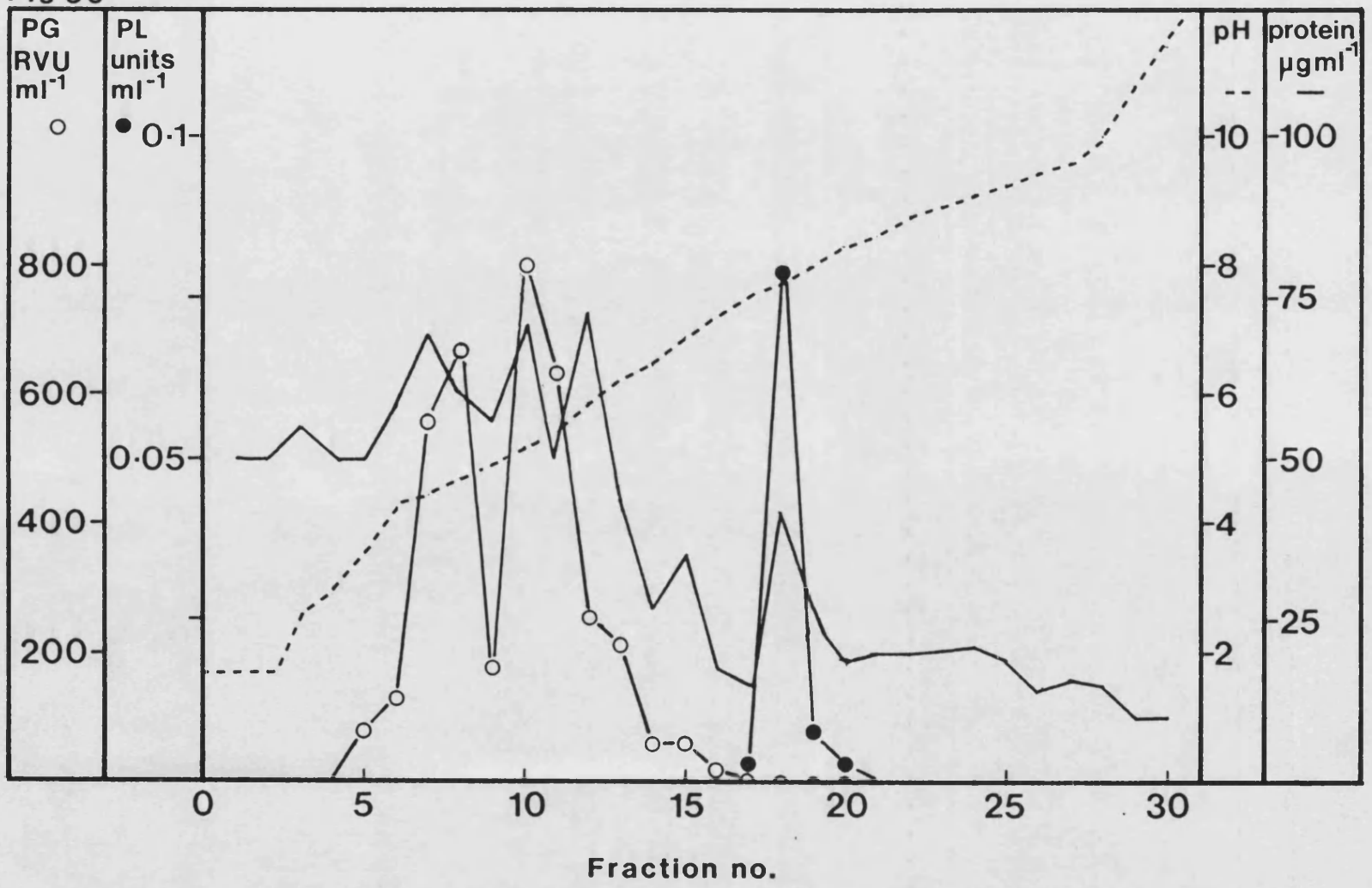

PG
RVU
ml-1
PL
units
ml-1
0·1
0·05
800
600
400
200
pH
10
8
6
4
2
protein
µgml-1
100
75
50
25
0
5
10
15
20
25
30
Fraction no.

Table 9. Production of PG and PL by *B. allii* isolates in 7 d pectin/ salts cultures (pH 5.0).

Isolate	pH	dry weight (mg)	PG (RVU)	PL (μmolml^{-1}min^{-1})
2068	5.5	67 ± 3.5	527 ± 8.5	0.038 ± 0.003
2070	5.6	60 ± 4.6	776 ±11.2	0.048 ± 0.003
3543	5.5	62 ± 3.8	554 ± 9.2	0.040 ± 0.004
3669	5.6	65 ± 5.7	506 ± 6.7	0.021 ± 0.004
3670	5.6	70 ± 4.8	612 ± 8.3	0.041 ± 0.004

Results are mean values from 3 shake cultures.

then dialysed and subjected to flat bed IEF followed by PG activity staining to determine differences in the isozyme profiles of the five isolates.

Of all the isolates, 2070 produced most PG and a high level of PL (Table 9). As 2070 also proved to be highly pathogenic on whole onion bulbs (Appendix 10) this isolate was chosen for further studies.

From Plate 2 it is clear that most of the PG activity is concentrated in the acidic region. There was a marked similarity between all of the isolates; each produced *c* 8 acidic PG isozymes including a highly active PG of pI 5.3-5.4. 3669 differs by having one additional PG of moderate activity in the basic region. 2068, 2070 and 3670 have almost identical profiles but differ in the relative amounts of PG and PL that they produce. A comparison of the PG zymograms of the *B. allii* isolates with those of *B. cinerea*, *B. fabae* and *B. squamosa* (Plate 3), revealed that each species possesses a distinct pattern.

2.1.3.1.2. Polygalacturonase isozyme profiles produced by *B. allii* on different carbon sources.

Previous results (Results and Discussion 1.1.2), have shown that the PG

Plate 2. Polygalacturonase isozyme profiles of five *B. allii* isolates as determined by flat bed isoelectric focusing.

(1) 2068; (2) 2070; (3) 3543; (4) 3669; (5) 3670.

Plate 3. Polygalacturonase isozyme profiles of *B. cinerea* (1), *B. fabae* (2) and *B. squamosa* (3) isolates as determined by flat bed isoelectric focusing.

Plate 2

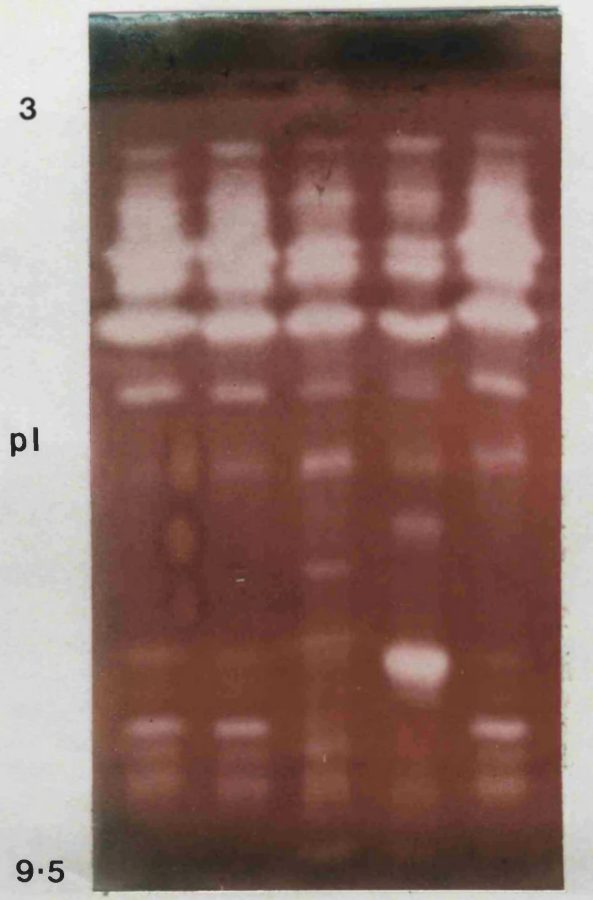


Plate 3

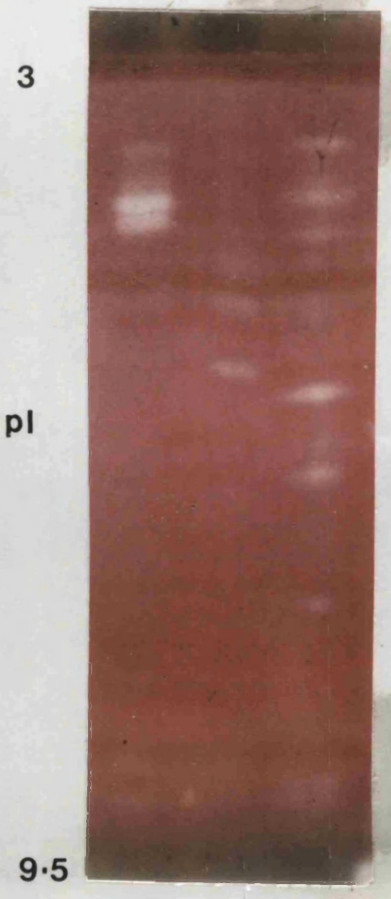


Plate 4

Isozyme profiles of polygalacturonase produced by *B. allii* on pectin, glucose, CMC and onion cell walls.

For densitometer analysis see Fig 37.

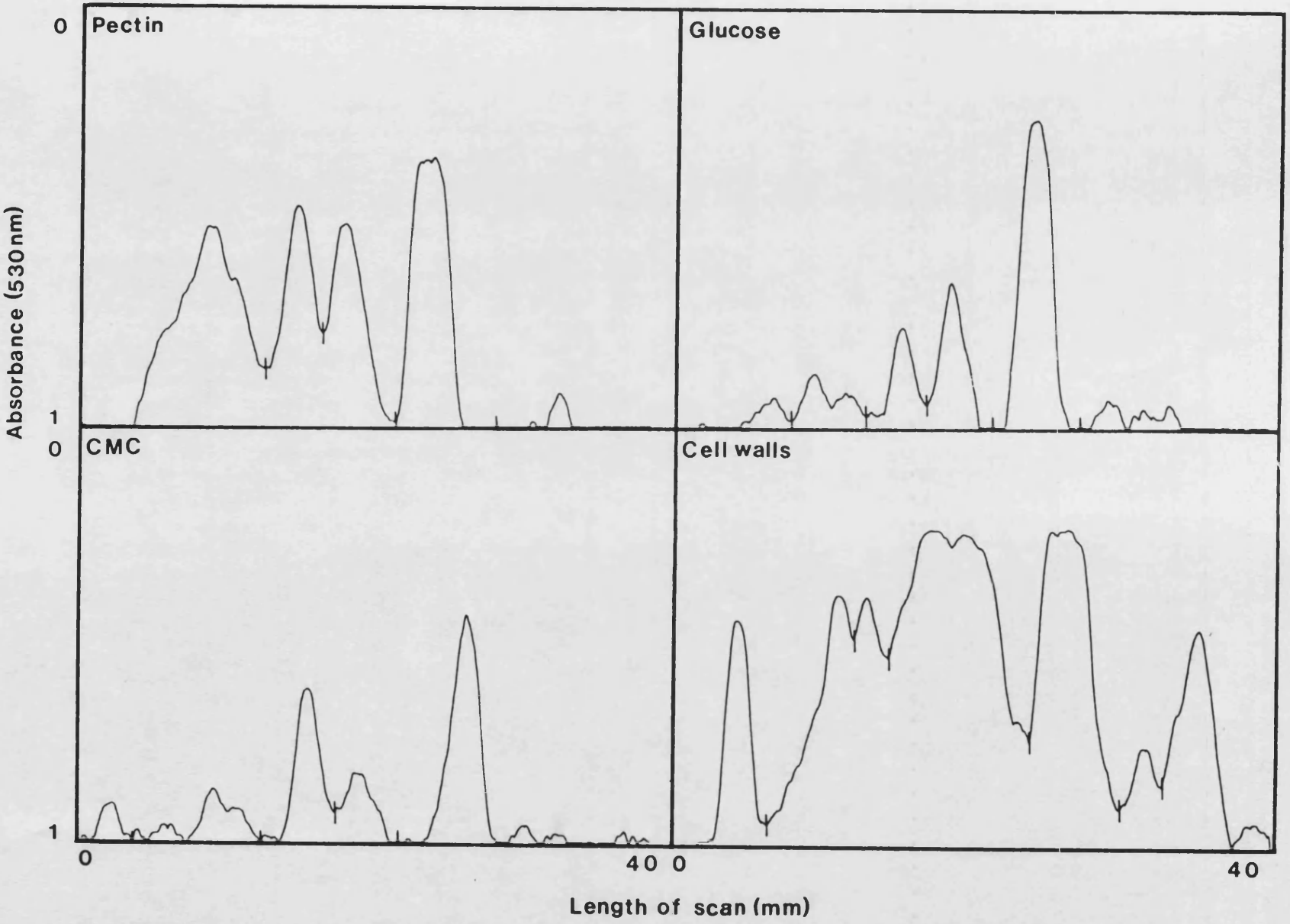
Fig 37. Densitometer profiles of *B. allii* polygalacturonase isozymes produced on pectin, glucose, CMC and onion cell walls, resolved by flat bed IEF.

The PG isozyme profiles on the flat bed PAGE gel shown on Plate 4 were scanned with a Joyce-Loebl densitometer (Chromoscan 3) at 530 nm.

Scan range: 40 mm (pI range 6.1-3.5)
Maximum absorbance: 1.0

Transmission peaks are indicative of PG activity, which is seen on the stained gels as clear zones in the red background.

Fig 37


Pectin
Glucose
CMC
Cell walls
0
1
0
1
Absorbance (530nm)
0
40
0
40
Length of scan (mm)

and PL of *B. allii* are induced on cell walls and pectin, providing the levels of sugars released are not high enough to cause repression of synthesis. PG and PL are also produced at basal levels in media containing CMC and glucose. Neither enzyme is produced constitutively and both are subject to CR. The PG activity released by *B. allii* under these different conditions may reflect altered overall levels of synthesis; alternatively changes in the combinations of PG isozymes could account for this variation.

PG produced in the shake flasks containing pectin, glucose, CMC and onion cell walls (Materials and Methods 1.3.1) was concentrated by ammonium sulphate precipitation and resolved by flat bed IEF as described above).

From Plate 4 and from the densitometer profiles (Fig 37) there is an overall correspondence of PG isozymes produced on cell walls and pectin. However, one PG isozyme of pI 3.4 was produced exclusively in cell wall cultures. The three isozymes detected in glucose and CMC filtrate may represent basal activity. The major PG isozyme (pI 5.4) is produced on all four media but five out of the nine most prominent isozymes are not apparent in the media lacking galacturonide. All of the isozymes would appear to be partly inducible as higher levels of each form are produced on pectin and cell walls.

2.1.3.2. Purification of PG and PL by column isoelectric focusing.

For separating PL and PG isozymes, concentrated filtrates of pectin/ salts cultures were routinely focused in a 110 ml LKB isoelectric focusing column (Materials and Methods 9.2.1).

The isozyme profile shown in Fig 36 is representative of three separate experiments. As indicated by results from flat bed IEF, PG activity can be attributed to several isozymes concentrated between pI's 3.5 and

6.5, and particularly to two major isozymes with pI's 5.4 and 4.8. The single form of PL could be readily separated from the major PG isozymes because of its relatively basic nature (pI 7.6).

Column IEF is clearly a powerful technique for the preparation of semi-pure isozymes, but the method lacks the fine resolution and sensitivity of flat-bed IEF (eg compare Fig 36 and Plate 4). In repeated experiments the maximum number of bands indicating PG activity on PAG plates was 13; 8 were detected regularly, the appearance of the others depended on the overall PG activity in the filtrate. Of the 8 major bands two were considerably larger than the others with pI's that conformed to the major peaks of PG activity found by fractionating filtrate by column IEF.

2.1.4. Purification and determination of molecular weight and Stokes' radius of *B. allii* PG and PL by Sephadex column chromatography.

Preparations of *B. allii* enzyme solutions were eluted with K phosphate buffer (pH 6.8, 0.1 M) through a Sephadex G-100 column as described in Materials and Methods 10, with the changes given in Table 10.

PG and PL have respective molecular weights of 43 000 and 26 500 daltons and can be readily separated by gel-filtration (Fig 38). Almost all of the PG activity was concentrated in a single peak; PL activity was found only in one fraction.

The component responsible for the predominant PG peak from concentrated pectin/salts filtrate in Fig 38, can be attributed (at least partially) to the major PG isozyme, pI 5.4 (Fig 39) detected by flat bed IEF (Plate 4; Results and Discussion 2.1.3.1.1). It is also important to note that the PG activity found in fractions of *c* pH 5.4 probably corresponded to a single form and not to two or more isozymes with identical pI's, based on the assumption that two separate isozymes will

Fig 38. Sephadex chromatography of *B. allii* polygalacturonase and pectin lyase.

Concentrated pectin/salts culture (pH 8.0, MES 0.05 M) filtrate was eluted down a column of Sephadex G-100. PG (○), PL (●), protein (-).

Column length: 54 cm.

Column diameter: 1.6 ml.

V_t: 108.6 ml.

V_o: 32 ml.

Elution rate: 3.6 ml h^{-1}.

Fraction size: 3.75 ml.

Total protein applied to column: 0.507 mg.

Total PG activity applied to column: 2 900 RVU.

Total PL activity applied to column: 1.24 µmol ml^{-1}.

Maximum PG activity (fraction 10): 348 RVU ml^{-1}.

Maximum PL activity (fraction 18): 0.104 µmol ml^{-1} min^{-1} ml^{-1} fraction.

Results are representative of three separate elutions.

Fig 38

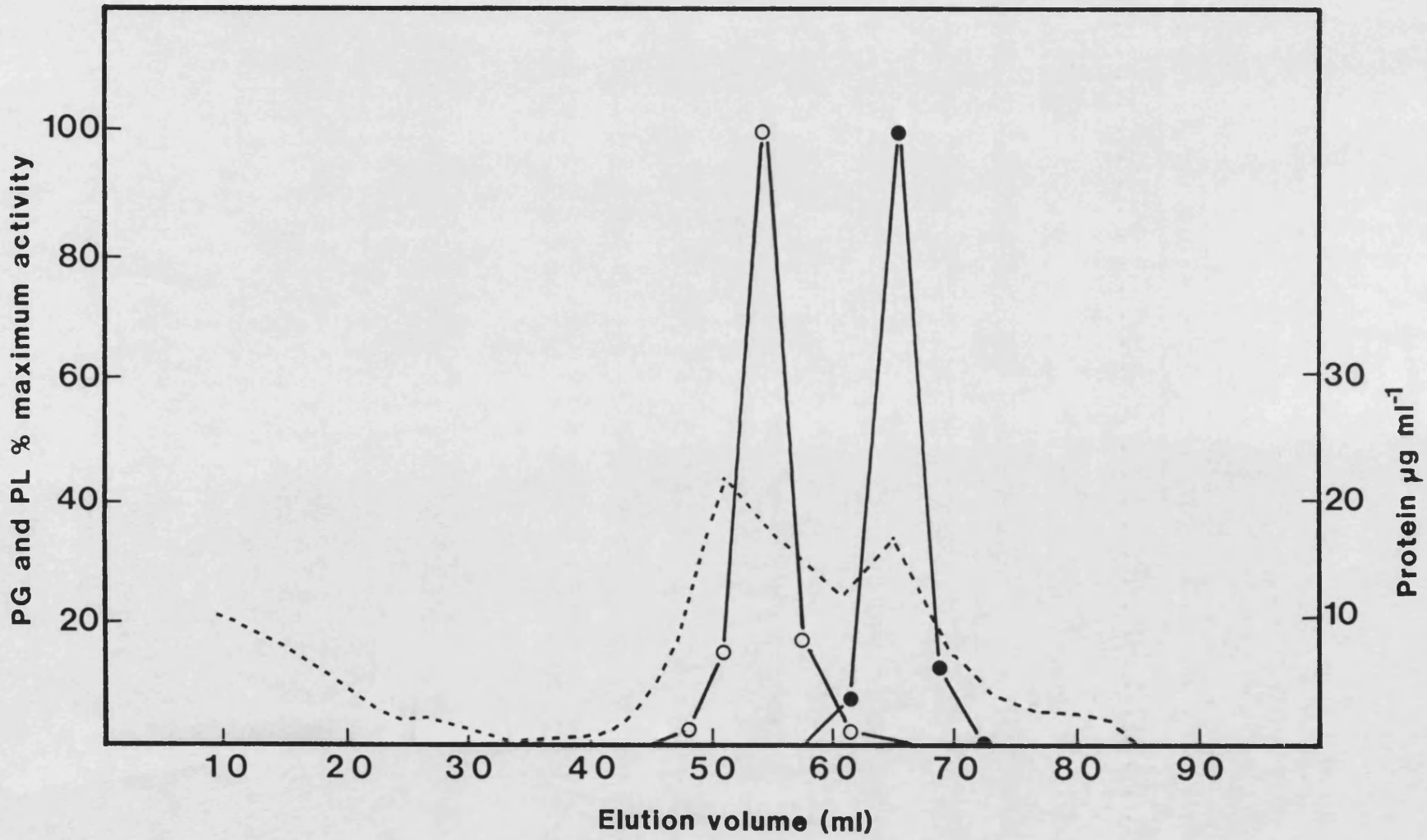

PG and PL % maximum activity
100
80
60
40
20
10
20
30
40
50
60
70
80
90
Elution volume (ml)
30
20
10
Protein µg ml-1

Fig 39. Sephadex chromatography of *B. allii* polygalacturonase isozyme (pI 5.4).

PG isozyme (pI 5.4) resolved by broad range IEF (pH 3-10) was eluted down a column of Sephadex G-100. PG (O), protein (-·-).

Column length: 54 cm.

Column diameter: 1.6 ml.

V_t: 108.6 ml.

V_o: 32 ml.

Elution rate: 3.6 ml h^{-1}.

Fraction size: 2.6 ml.

Total protein applied to column: 0.096.

Total PG activity applied to column: 960 RVU

Maximum PG activity: 116 RVU ml^{-1} fraction.

Results are representative of three separate elutions.

Fig 39

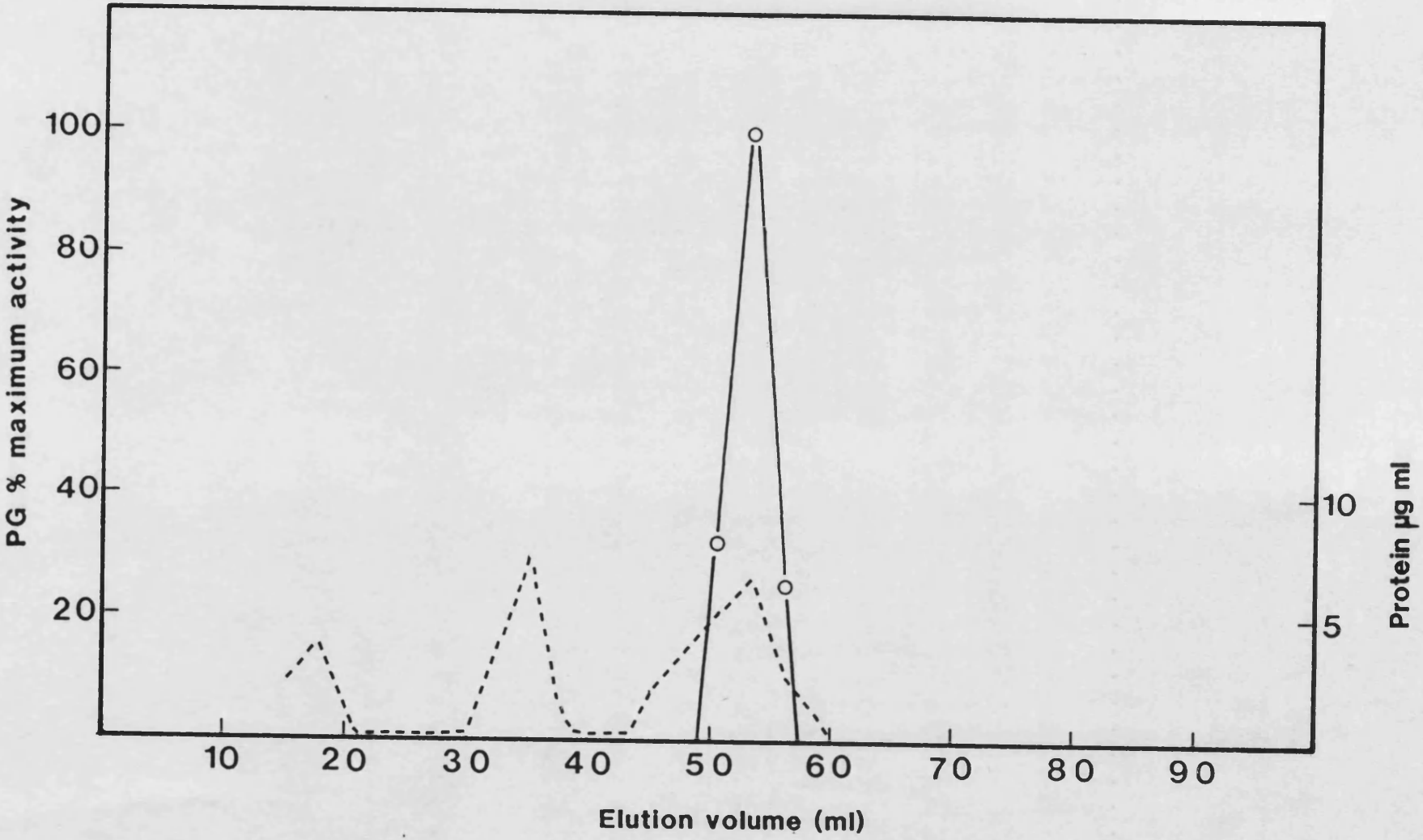

PG % maximum activity
100
80
60
40
20
10
5
Protein µg ml
10
20
30
40
50
60
70
80
90
Elution volume (ml)

Fig 40. Sephadex chromatography of *B. allii* pectin lyase.

PL (pI 7.6) resolved by broad range IEF (pH 3-10) was eluted down a column of Sephadex G-100. PL (●), protein ←→.

Column length: 54 cm.

Column diameter: 1.6 ml.

V_t: 108.6 ml.

V_o: 32 ml.

Elution rate: 3.6 ml h^{-1}.

Fraction size: 3.75 ml.

Total protein applied to column: 0.021 mg

Total PL activity applied to column: 0.15 µmol ml^{-1}.

Maximum PL activity (fraction 18): 0.01 µmol ml^{-1} min^{-1} ml^{-1} fraction.

Results are representative of three separate elutions.

Fig 40

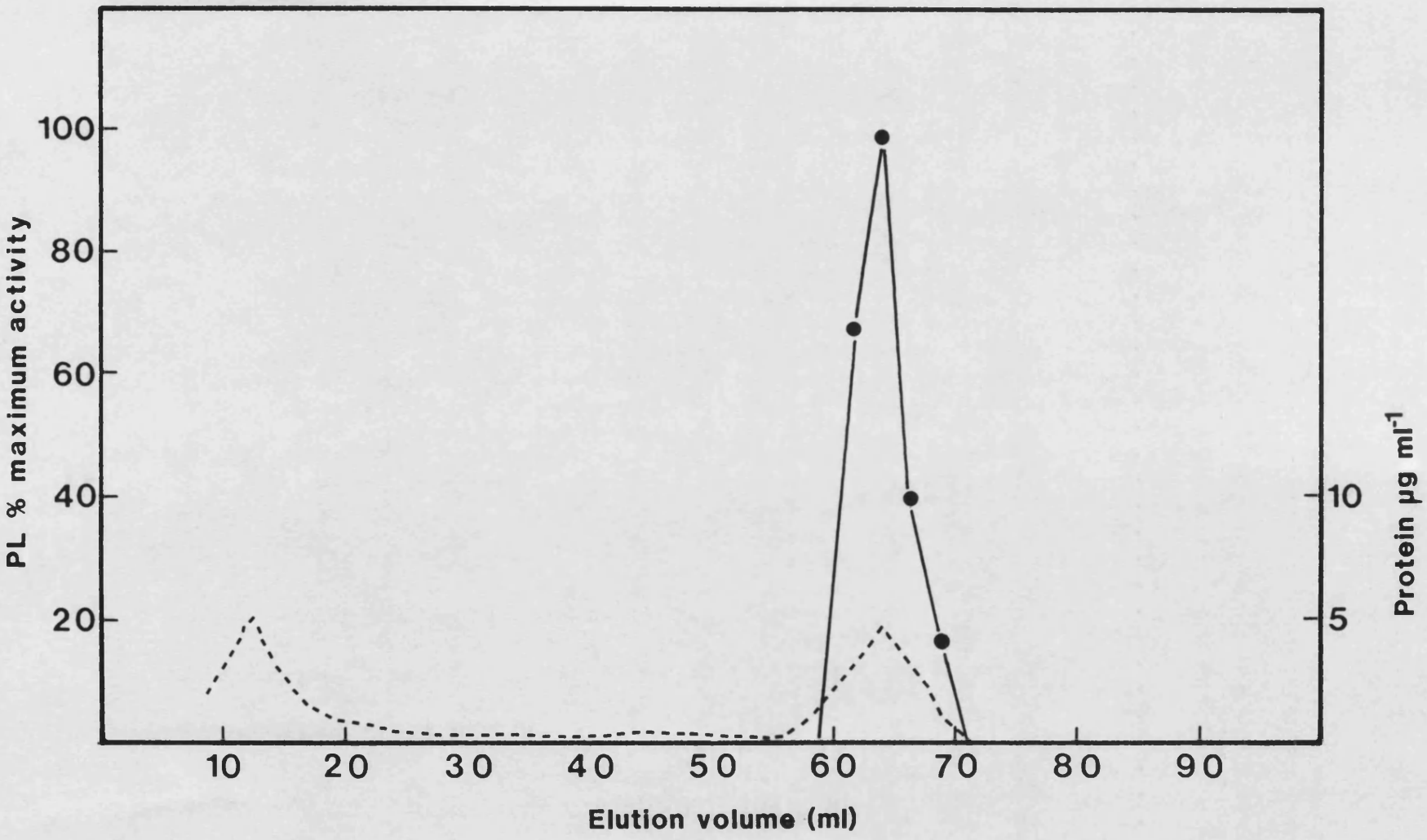

100
80
60
40
20
PL % maximum activity
10
5
Protein µg ml-1
10
20
30
40
50
60
70
80
90
Elution volume (ml)

Table 10. Molecular weights and Stokes' radii of *B. allii* PG and PL as determined by gel-filtration (see Figs 38-40). *a*

Sample	Molecular weight (daltons) PG	PL	Stokes' radius (Å) *b* PG	PL
Conc. pectin/salts culture filtrate	43 500	26 000	30.7	22.4
IEF fraction 10 pH 5.4	43 000	-	30.5	-
IEF fraction 18 pH 7.7	-	27 000	-	22.7

a Column diameter, 1.6 cm; elution rate, 3.6 ml h^{-1}, fraction size, 3.6 ml (see Figs 38-40).
b Molecular weights and Stokes' radii estimated from elution form, as determined by IEF and constants (K_{av}) of marker proteins and peaks of PG and PL activity for this column (Materials and Methods 10).

not share the same pI and molecular weight. PL activity also corresponds to a single gel-filtration (Fig 40). Allowing for experimental error the MW and R_m values for PG and PL obtained by this method remained consistent with 3 separate elutions.

2.1.5. Summary of purification of *B. allii* PG and PL.

After dialysis of culture filtrate PG and PL activity increased, whether expressed as overall or as specific activity. Ammonium sulphate precipitation proved to be satisfactory for initially concentrating the enzymes and most of the low molecular weight contaminating protein was removed, although there were 3-fold losses in activity. IEF was an essential step in obtaining semi-pure isozymes but the extensive dialysis and focusing time caused considerable losses of both PG and PL activity, and particularly the lyase. Sephadex chromatography was an excellent method for separating PL as it is much smaller than the PG isozymes; in addition the yield was very much higher than from purification by IEF. PG and PL isozyme fractions from IEF column could be further purified *c* 3-fold by passing through Sephadex (Table 11).

Table 11. Summary of purification of *B. allii* PG and PL.

	Total				Specific activity	
	volume (ml)	protein (mg)	PG (RVU)	PL (μmol ml^{-1} min^{-1})	PG (protein mg^{-1})	PL
Pectin/salts culture filtrate	600	207.3	75 000	18.0	362	0.087
Dialysed filtrate	620	94.6	89 900	26.0	950	0.275
Amm. sulph. Concentrate	18	4.5	26 100	11.2	5 800	2.48
IEF of ammonium sulphate concentrate						
Amm. sulph. conc.	14	3.5	20 300	8.67	5 800	2.48
Fract. pH 5.4	3.7	0.30	3 000	-	10 000	-
Fract. pH 7.6	3.7	0.09	-	0.29	-	3.22
Sephadex chromatography of ammonium sulphate concentrate						
Amm. sulph. conc.	2	0.507	2 900	1.24	5 720	2.48
Fract. 10	3.6	0.065	1 253	-	19 336	-
Fract. 18	3.6	0.050	15	0.375	298	7.44
Sephadex chromatography of IEF fractions						
IEF Fract. pH 5.4	1.2	0.096	960	-	10 000	-
Fract. 10	2.6	0.034	305	-	8 970	-
IEF Fract. pH 7.6	1.5	0.021	-	0.15	-	7.44
Fract, 18	2.5	0.011	-	0.010	-	0.91

2.1.6. Mode of action of *B. allii* PG and PL on defined substrates.

2.1.6.1. The relationship between degradation and viscosity reduction of substrate.

To determine whether cleavage of pectic substrates by PG and PL of *B. allii* is random (*endo*) or terminal (*exo*), the relationship between substrate viscosity and substrate breakdown was examined. *Endo*-enzymes are usually reported to reduce substrate viscosity by *c* 50 % by cleaving *c* 1-2 % of glycosiduronic bonds (Nasuno & Starr, 1967).

2.1.6.1.1. Polygalacturonase.

PG activity was determined by viscometry and by TBA in equivalent reaction mixtures. From curves of viscosity reduction against time, t_{50}

Table 12. The relationship between viscosity reduction and substrate hydrolysis by PG of *B. allii* (see Figs 41 and 42).

PG frompectin/salts(*a*) cultures, pH 5.0	time (min) 10	20	40	60	120
% decrease in viscosity(*c*)	50	62.5	77	81	85
% hydrolysis(*d*)	3.2	6	11.2	16	26.6
PG, major isozyme (*b*) pI 5.4					
% decrease in viscosity (*c*)	44	60	74	80	90
% hydrolysis (*d*)	0	0	0.16	5.7	8.3

a PG from pectin/salts (pH 5.0) medium was partially purified by ammonium sulphate precipitation.
b PG isozyme (pI 5.4) from column IEF fractionation.
c Viscometric assay: 2 ml of PG solution were reacted with 8 ml of NAPP (pH 5.0, 0.1 M MES). Decrease in viscosity determined in Technico viscometers at 25°C.
d Substrate hydrolysis calculated on the basis of 78 % (w/w) galacturonide in substrate. Concentration of GALA released was determined by boiling 1 ml samples from similar reaction mixtures with 1 ml TBA (see Materials and Methods 6.2).

Fig 41. Release of GALA (●) and reduction in viscosity (O) in NAPP (1 % w/v; pH 5.0, citrate 0.1 M) by *B. allii* polygalacturonase from pectin/salts (pH 5.0, MES 0.05 M) culture filtrate incubated at 25°C (see Table 12 for conditions).

Fig 42. Release of GALA (●) and reduction in viscosity (O) in NAPP (1 % w/v; pH 5.0, citrate 0.1 M) by *B. allii* PG isozyme (pI 5.4) incubated at 25°C (see Table 12).

Results are representative of four separate replicate treatments.

Fig 41

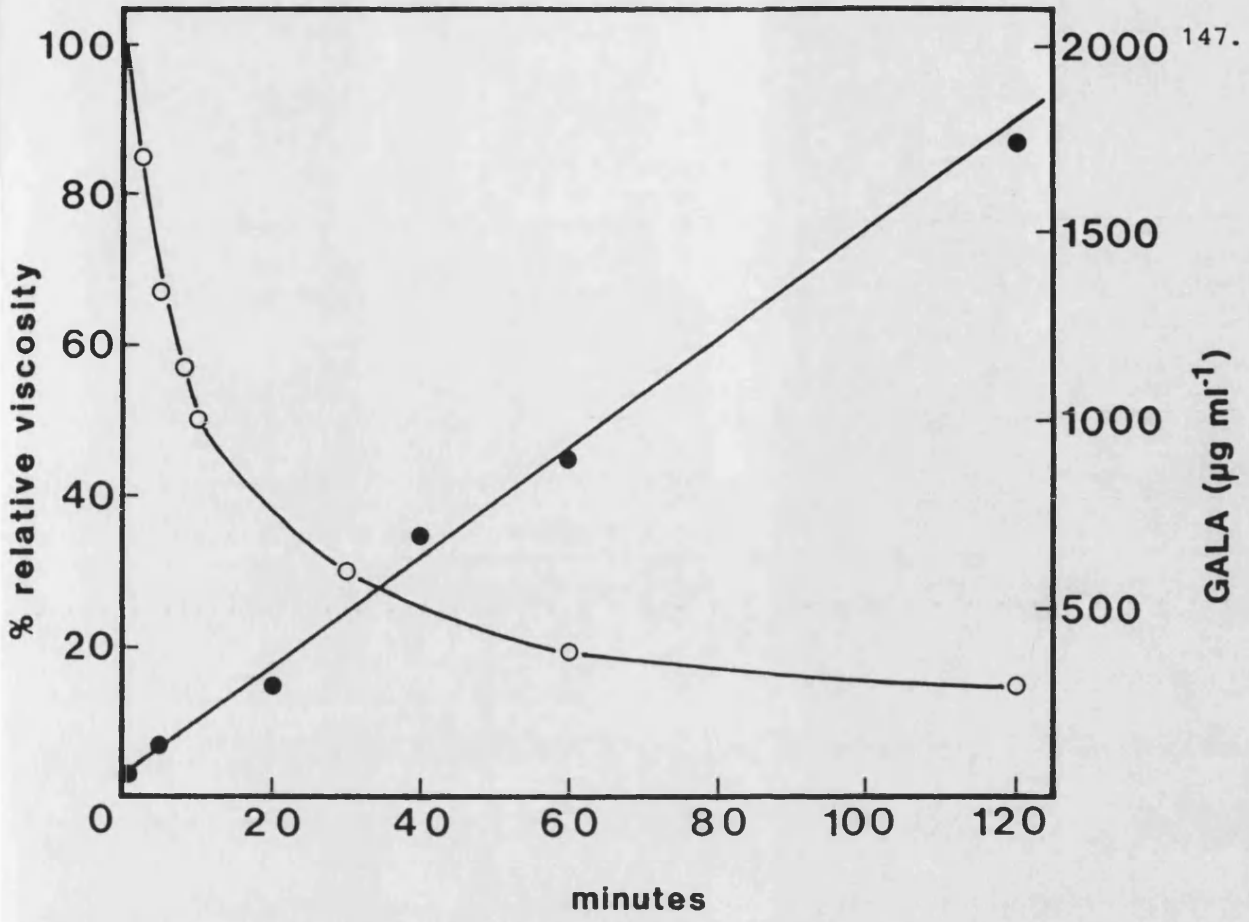
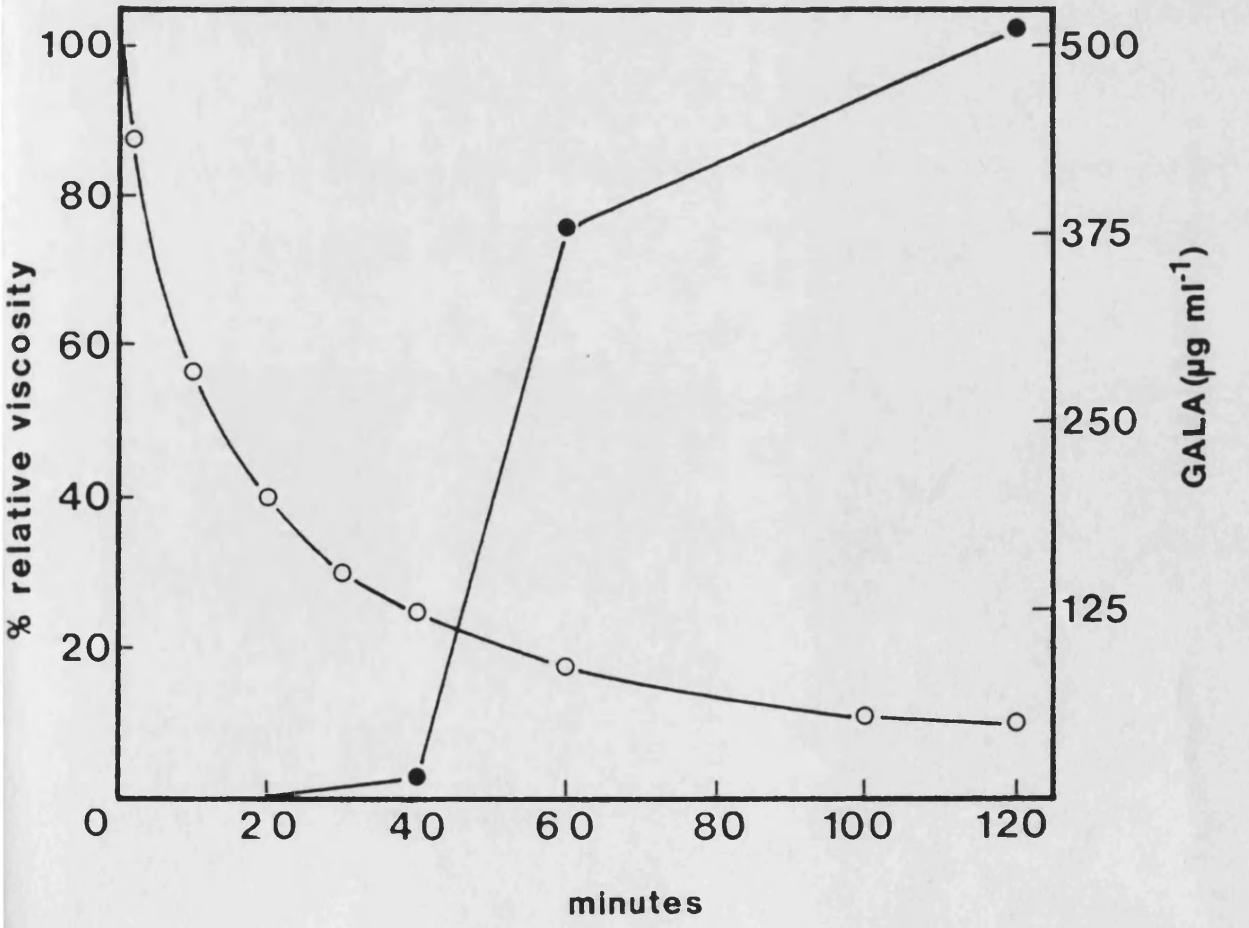

100
80
60
40
20
0
20
40
60
80
100
120
2000
1500
1000
500
% relative viscosity
GALA (µg ml-1)
minutes

Fig 42


100
80
60
40
20
0
20
40
60
80
100
120
500
375
250
125
% relative viscosity
GALA (µg ml-1)
minutes

values were obtained. From curves of galacturonide release against time in similar reaction mixtures, levels of substrate degradation corresponding to t_{50} were calculated for the major PG isozyme (pI 5.4) (Fig 41) and PG in crude, dialysed pectin/salts cultures (pH 5.0), (Fig 42). The degree of degradation and the progressive decrease of substrate viscosity achieved by PG during the incubation was determined for three replicate treatments (Table 12).

A 50 % reduction in the relative viscosity of NAPP by PG from pectin/salts cultures was accompanied by 3.2 % substrate hydrolysis which suggests that degradation is not exclusively random and that a degree of terminal cleavage is taking place. The corresponding figure for NAPP degradation by the principal PG isozyme was <0.16 % which would imply that it degrades NAPP by random action. A rapid increase in substrate hydrolysis occurred between 40 and 60 min while the accompanying decrease in viscosity was only 16 %. This suggests that either there was an undetected exo-PG present in the IEF fraction containing the major endo-PG or that this PG isozyme is a multiple enzyme that is capable of terminal cleavage in addition to random cleavage.

2.1.6.1.2. Pectin lyase.

PL activity was determined by viscometry and by TBA in identical reaction mixtures. As for PG above, t_{50} values were obtained from curves of viscosity reduction against time. Degrees of substrate degradation at t_{50} and at points during the reaction were calculated (Table 13) from curves of galacturonide release against time (Fig 43).

PL behaved as an exo-enzyme. Substrate lysis was accompanied by a relatively slow rate of viscosity reduction (Fig 43 and Table 13). When c 2.5 of the substrate bonds had been cleaved the corresponding decrease in viscosity was only c 8 %. Release of UGALA by PL followed a similar pattern when pectin (0.1 %) degradation was measured by UV

Fig 43. Release of UGALA (●) and reduction in viscosity (○) in pectin (1 % w/v; pH 8.0, HEPES 0.05 M) by *B. allii* PL incubated at 25°C (Table 13).

Results are representative of four separate replicate treatments.

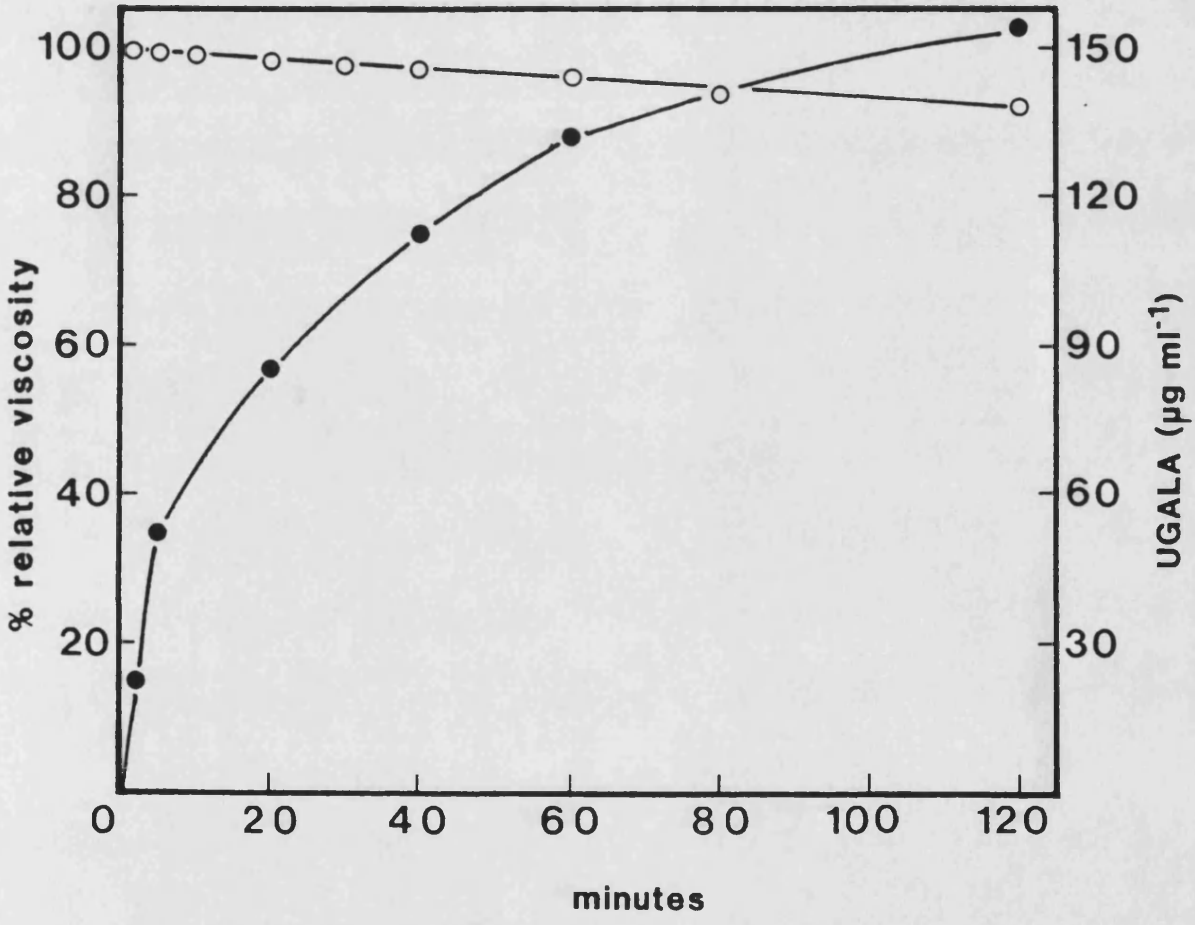


Table 13. The relatonship between viscosity reduction and substrate lysis by PL of *B. allii* (see Fig 43).

	time (min)				
PL isozyme (pI 7.6) (*a*)	10	20	40	60	120
% decrease in viscosity (*b*)	1	1.5	1.8	4	8
% lysis (*c*)	1.2	1.5	1.8	2.1	2.5

a PL isozyme (pI 7.6) prepared by column IEF fractionation.
b Viscometric assay: 2 ml of PL solution were reacted with 8 ml of 1 % pectin (pH 8.0, 0.05 M HEPES). Decrease in viscosity was determined in Technico viscometers at 25°C.
c Substrate lysis calculated on the basis of 78 % (w/w) galacturonide in substrate. Concentration of UGALA released was determined by boiling 1 ml samples from similar reaction mixtures with TBA. Activity on pectin (0.1 %) as determined by UV absorbtion was *c* 0.05 μmol min^{-1} ml^{-1}.

absorption; the reaction which is initially linear decreases over time and eventually becomes static. Enhancement of activity by removal of of low molecular weight products from culture filtrates by dialysis suggests that oligomers based on UGALA inhibit PL activity and eventually cause the decline in the rate of substrate degradation.

2.1.6.2. Mechanisms of substrate degradation.

The mono- and oligo-galacturonide composition of reaction mixtures was examined at intervals by TLC to determine how the pectic subsrates were degraded by PG and PL of *B. allii*. To prevent blistering of the TLC plates and tailing of the samples, substrate and buffer concentrations were kept to a minimum (see Materials and Methods 8).

2.1.6.2.1. Polygalacturonase.

PG of similar activity to that used in comparing rates of substrate hydrolysis and viscosity was reacted with NAPP at 25°C. The reaction mixture contained:

8 ml NAPP 0.1 % (w/v) pH 5.0 (MES 0.05 M)

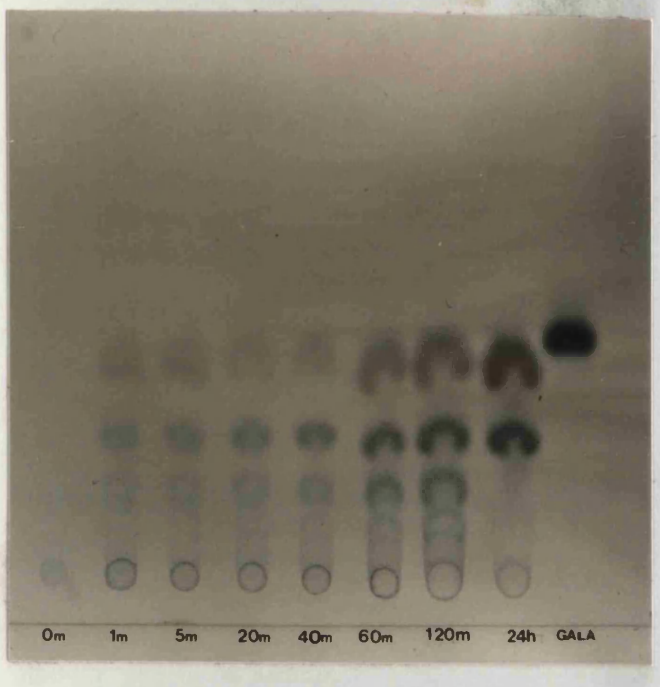
2 ml PG (100 RVU)

Samples were removed at intervals and reactions were stopped by heating at 100°C for 5 min. Samples were applied to TLC plates and spots were visualised with aniline-diphenylamine phosphate as outlined in Materials and Methods 8. GALA oligomers were identified by their relative mobility compared with mono-galacturonic acid which was used as a marker. R_{GALA} values obtained for GALA oligomers closely compare with those of Cooper (1974). Although NAPP degradation occurred in a predominantly random manner, as determined by the relatively fast decrease in viscosity and simultaneous slow rate of substrate hydrolysis, low molecular weight oligomers were released during the initial stages of degradation which is indicative of terminal cleavage of the substrate.

The NAPP degradation products released by crude PG contained only traces of oligomers larger than tri-galacturonic acid. The high proportion of mono-, di- and tri-galacturonic acids released in the early stages of hydrolysis indicated the presence of an exo-PG isozyme(s). Monomer and dimer concentrations gradually rose throughout the reaction, as the trimer almost disappeared after 1 h. The monomer and the dimer, are therefore, at least partially the products of trimer cleavage.

The major PG isozyme (pI 5.4) also released mono-, di- and tri-galacturonic acid during the initial stages of substrate degradation. However tetra-, penta- and hexa-galacturonic acids were also detected on TLC plates even after 2 h incubation (Plate 5) and the presence of these larger oligomers was indicative of random cleavage. Monomer and dimer levels did not increase significantly until trimer levels had risen after *c* 40-60 min. The increase of these three products at this time coincided with the sudden rise in total GALA residues during NAPP degradation as detected by TBA (Fig 2). It would seem that the rate at

Plate 5



Thin layer chromatograms of uronide degradation products released by *B. allii* PG isozyme (pI 5.4) from NAPP.

Samples removed from reaction mixture after 1, 5, 20, 40, 60, 120 min and 24 h. Mono-GALA was applied as as a standard.
See Table 12 and Fig 42 for experimental conditions and Materials and Methods 8. for details on TLC.

which this apparent exo- activity occurred was dependent on the levels of trimer that were available in the reaction mixture. Di-galacturonic acid was the predominant product throughout the reaction until at 24 h it was slightly exceeded by higher levels of the monomer (as determined by densitometry). As the dimer and the monomer were not released at equivalent levels during the initial stages of the reaction the origin of the dimer was probably not the trimer; but from the breakdown of oligomers >hexamer as they did not appear on the TLC plates.

The major PG isozyme behaved as an endo- acting enzyme with a further capacity to degrade GALA oligomers as small as the trimer. In contrast, an unknown exo-PG, present in the reaction mixture may have been responsible for the degradation of the smaller oligomers.

2.1.6.2.2. Pectin lyase.

PL purified by IEF (0.05 μmol ml^{-1} min^{-1}, of similar activity to that used in comparing rates of substrate hydrolysis and viscosity) was reacted with pectin at 25°C. The reaction mixture contained:

8 ml pectin 0.1 % (w/v) pH 8.0 (HEPES 0.05 M)

2 ml PL (0.25 μmol ml^{-1} min^{-1})

Samples were removed at intervals and reactions stopped by heating at 100°C for 5 min (as above). Samples were analysed by TLC and by paper chromatography as outlined in Materials and Methods 7 and 8.

Faint blue spots were seen on TLC plates treated with aniline-diphenylamine phosphate. From their R_{GAL} values the spots corresponded to mono and di-galacturonic acid. Similar plates treated with the TBA reagent revealed red spots (at 365 nm), of equivalent R_{GAL} values. This confirmed that the spots were unsaturated mono- and di-galacturonic acid. R_{GAL} values for mono- and di-unsaturated galacturonic acid) were 0.82 and 0.6 respectively. Furthermore, paper

chromatograms stained with silver nitrate also revealed two spots that were probably mono- and di-UGALA with R_{GAL} values of 0.79 and 0.52 which closely agreed with the figures obtained by Collmer *et al.* (1982a). Both sugars were present in the reaction mixture upto 2 h, but at 24 h only the monomer was detected. These results provided further evidence that this PL was an *exo*-acting enzyme.

2.1.7. PG and PL isozymes in onion tissue infected with *B. allii*.

B. allii produces 2 major isozymes of endo-PG *in vitro* as determined by IEF of pectin/salts culture filtrate (Fig 44). Either of these 2 isozymes or a combination of these and some of the remaining 11 (so far detected) may be involved in disease development. Alternatively a novel range of isozymes may be found *in vivo*. Extracts from 48 h lesions were fractionated by column IEF to show if the PG activity detected *in vivo* corresponded to the isozymes that were produced *in vitro*. The summary of purification is presented in Table 14.

The IEF profile of PG activity in lesions (Fig 44) closely resembled the profile obtained from pectin/salts culture filtrate (Fig 36).

Table 14. Summary of purification of PG isozyme pI 5.4 and PL from lesion extract by IEF (see Fig 44).

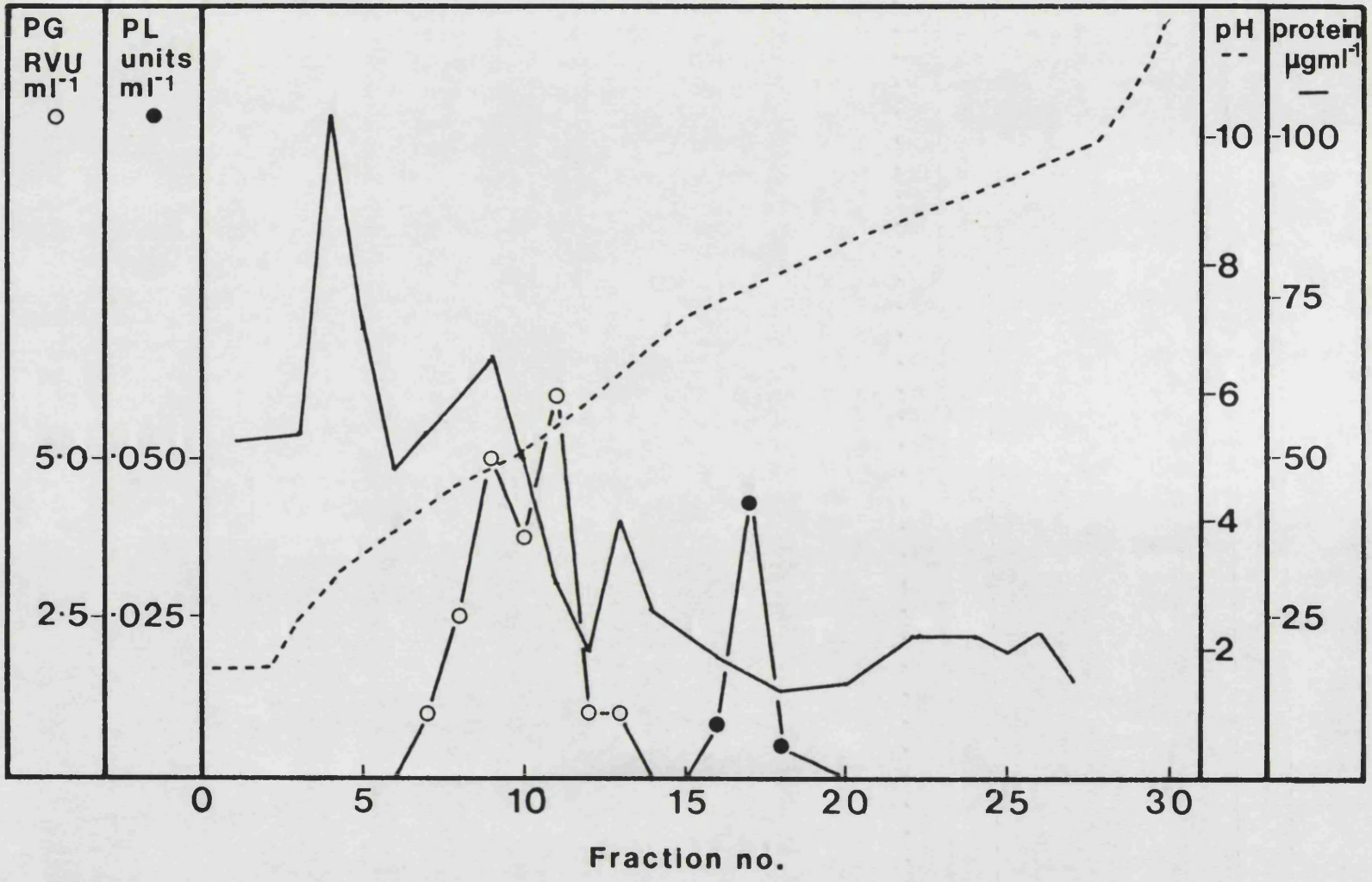
Source	Total volume (ml)	Protein (mg)	PG (RVU)	PL (μmol ml^{-1} min^{-1})	Specific activity (mg^{-1}) PG	PL
Amm. sulph. ppt.	18	2.54 ±0.60	90.2 ±4.1	1.5 ±0.2	37.1 ±5.1	0.6 ±0.04
IEF fract. pH 5.4	4.4	0.124 ±0.013	24.2 ±4.2	-	192.0 ±8.4	-
IEF fract. pH 7.6	4.4	0.066 ±0.007	-	0.19 ±0.04	-	2.85 ±0.02

Results are mean values of two separate experiments.

Fig 44. Broad range isoelectric focusing (pH 3-10) of polygalacturonase and pectin lyase from onion tissue infected with *B. allii* (48 h). PG (O), PL (●), protein (—), pH (- -).

Maximum PG activity: 5.9 RVU ml^{-1}.
Maximum PL activity: 0.0425 μmol^{-1} ml^{-1} min^{-1} ml^{-1}.

Fig 44


PG
RVU
ml⁻¹
PL
units
ml⁻¹
pH
protein
µgml⁻¹
5·0
·050
2·5
·025
10
8
6
4
2
100
75
50
25
0
5
10
15
20
25
30
Fraction no.

The two major PG isozymes (pI's 4.7 and 5.4) and the single PL enzyme were present. However, the amount of PL detected *in vivo* (Table 14) relative to the level of PG (isozyme, pI 5.4), was 80 x higher than *in vitro* in pectin/salts cultures (Table 11). This marked differential effect cannot apparently be explained by the influence of pH as the pH differences between *in vitro* and *in vivo* were minimal (Results and Discussion 1.5). From the specific activities, column IEF purified PG and PL by 5 fold (Table 14).

The PG levels obtained from lesion extracts were too low to obtain good clearing zones in NAPP on IEF gels, but faint clearing was evident in the region associated with the major PG isozyme (pI 5.4).

2.1.8. Maceration and cell killing of onion tissue by *B. allii* PG and PL *in vitro*.

There are many examples where PG and PL produced by plant pathogens have been shown *in vitro* to kill plant cells in associating with tissue maceration (Basham & Bateman, 1976). Furthermore, based on microscopic evidence, the PG of *B. allii* has been implicated as a cell killing factor *in vivo* (Stewart & Mansfield, 1985a). In view of these findings and the necrotrophic mode of parasitism by *B. allii*, the effects of PG and PL on living tissue were investigated.

Reaction mixtures contained enzyme, buffer and squares (5 x 5 mm) of inner epidermis from mid-bulb scales, as described in Materials and Methods 18. The cell killing effects of PG isozyme (pI 5.4), PL and dialysed pectin/salts culture filtrate containing all the *B. allii* pectic enzymes was tested by counting the number of surviving cells that were able to retain the vital stain neutral red in plasmolysing conditions.

The major PG isozyme (pI 5.4) and the pectin/salts culture filtrate, adjusted to similar PG activity, caused the death of onion cells after

Table 15. Maceration of onion tissue and release of degradation products by PG and PL of *B. allii* (see Figs 45 and 46).

	Maceration *a*	Degradation products ($\mu g\ ml^{-1}$) *b*	Enzyme activities during incubation ml^{-1} *c*		
			0 h	12 h	48 h
PG (pI 5.4) (RVU) *d*	3 ±0.4	150 ±32	500	0	0
Crude PG (RVU) *e*	3 ±0.35	260 ±46	500	300 ±67	50 ±12
PL (μmol ml^{-1} min^{-1}) *f*	0	60 ±13	0.1	0	0

Mean results of 3 replicate treatments.

a Maceration expressed in arbitrary units 0-4; tested after 48 h incubation. No loss of tissue coherence was detected in the controls or in treatments after 12 or 24 h.
b Estimated in reaction mixtures by TBA after 48 h.
c Enzyme activities ml^{-1} as tested in samples removed from 10 ml reaction mixture containing enzyme, buffer and 40 epidermal squares.
d PG isozyme (pI 5.4) from column IEF. Reaction held at pH 5.0 (MES, 0.05 M).
e Dialysed pectin/salts culture filtrate containing the full complement of pectic enzymes (pH 7.0, HEPES 0.05 M). PL activity 0.12 μmol min^{-1} ml^{-1} min^{-1}.
f PL (pI 7.6) as prepared by column IEF, pH 8.0 (HEPES, 0.05 M).

4 h of incubation but was not correlated with tissue maceration which (Fig 45), as determined by vital staining with neutral red, was strongly correlated with K^+ leakage (Fig 46). K^+ leakage occurred was only detectable after 48 h. The PG isozyme and crude PG (pI 5.4) solubilised GALA from tissue.

PL which is an exo-enzyme failed to macerate the onion tissue (Table 15), although it did cause limited cell leakage and degradation of cell wall galacturonan, as measured by the release of UGALA; however PL did not cause cell death.

Neither PG nor PL activity was detected after 12 h incubation, presumably as a result of binding to and/or inactivation by host tissue. However PG activity remained in treatments containing PG from

Fig 45. Loss of viability of onion epidermal cells by incubation with polygalacturonase and pectin lyase of *B. allii*.

5 x 5 mm epidermal squares, removed from mid bulb onion scales were incubated with concentrated pectin/salts culture filtrate (○), semi-pure PG isozyme (pI 5.4) (◇) and semi-pure PL (△) at 25°C. Cell viability was determined by neutral red staining under plasmolysing conditions (KNO_3, pH 8.0; see Table 15). Control treatments contained buffer and autoclaved enzyme (●, ◆ and ▲).

Initial PG isozyme (pI 5.4) activity: 500 RVU ml^{-1}, buffered at pH 5.0 (MES, 0.05 M).

Initial PL activity: 0.1 μmol ml^{-1}, buffered at pH 8.0 (HEPES, 0.05 M).

Initial PG activity in pectin/salts concentrate: 500 RVU ml^{-1}, buffered at pH 7.0 (HEPES, 0.05 M).

Mean results of three replicate experiments.

Fig 46. Release of K^+ ions from onion epidermal cells by polygalacturonase and pectin lyase of *B. allii*.

K^+ concentration was determined in samples of filtrate from the onion epidermal squares incubated with crude PG (○), semi-pure PG isozyme (pI 5.4) (◇) and semi-pure PL (△) under the conditions described in Fig 45. Control treatments contained buffer and autoclaved enzyme (●, ◆ and ▲).

Mean results of three replicate experiments.

Fig 45

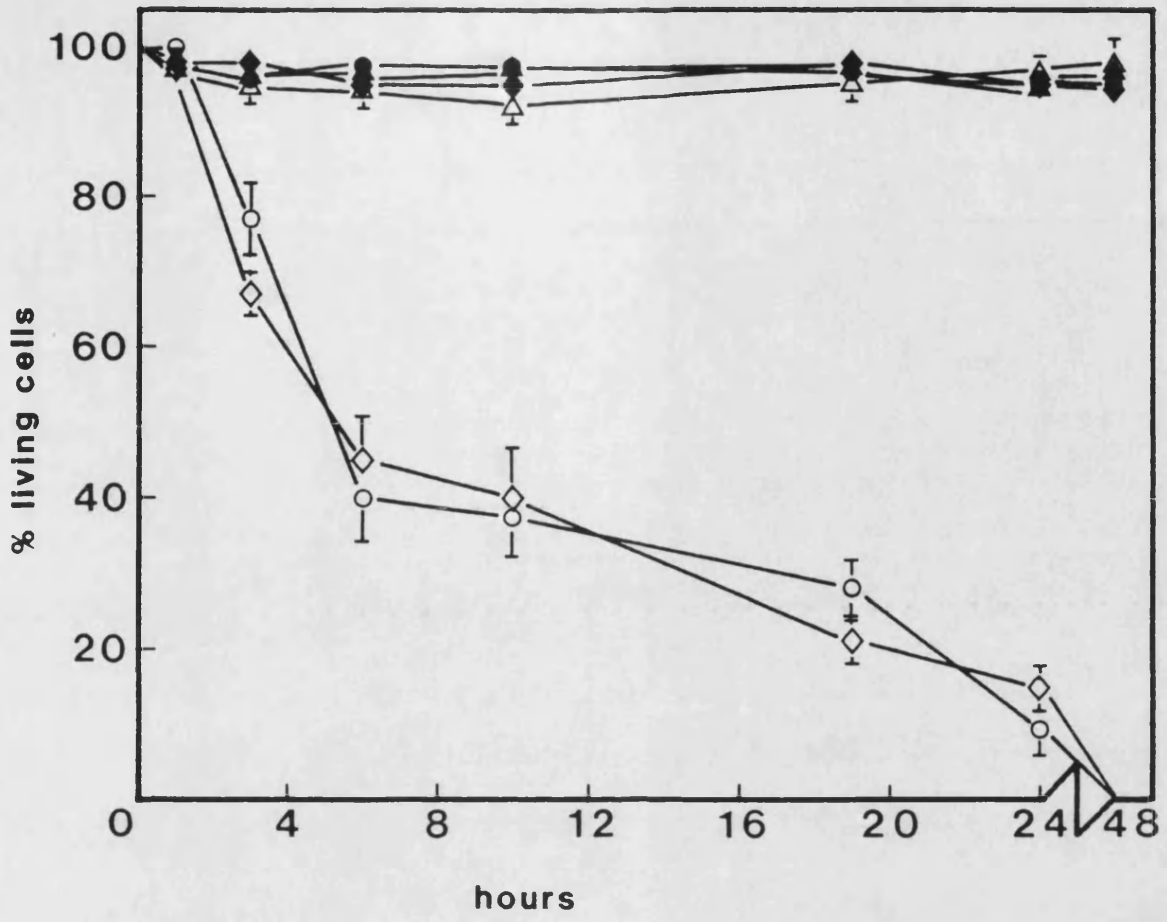
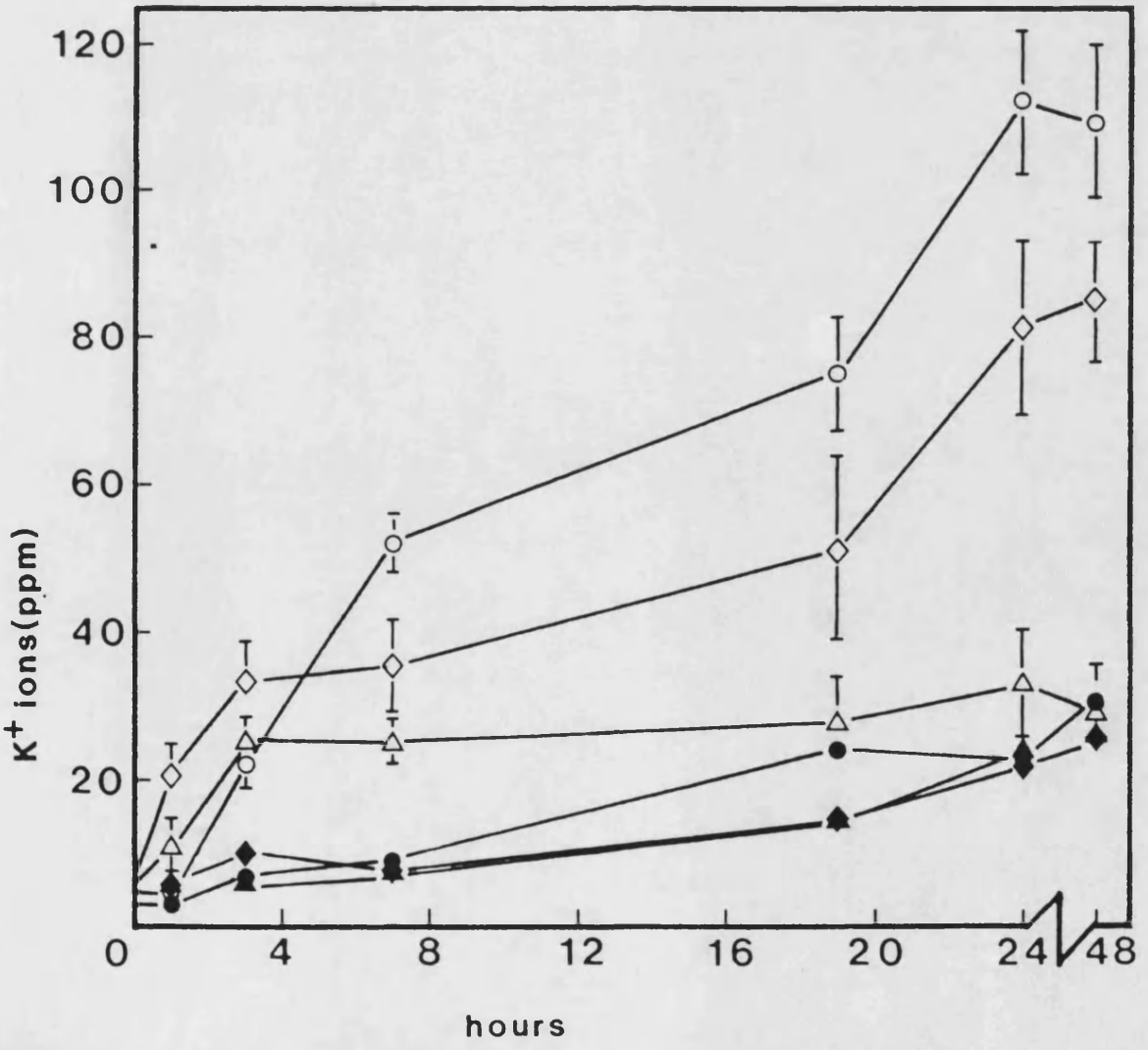

100
80
60
40
20
0
4
8
12
16
20
24
48
% living cells
hours

Fig 46


120
100
80
60
40
20
0
4
8
12
16
20
24
48
K+ ions(ppm)
hours

culture filtrate; therefore it would seem that some PG isozymes were less labile to effects of host tissue.

Cell leakage and death was not apparent in control treatments containing autoclaved enzymes which discounted possible toxic buffer effects or other non-labile toxic factors produced in culture by *B. allii*. Furthermore, differences in reaction mixture pH had little effect on cell leakage and killing.

2.2. Resolution of *V. albo-atrum* polygalacturonase and pectin lyase isozymes by isoelectric focusing.

VAA produces multiple forms of PG and PL (Cooper, *et al.*, 1978) as resolved by broad range column IEF. However recent evidence from flat bed IEF suggests the existence of many more PG isozymes (Mohan & Ride, 1984). It was essential to determine the total number of isozymes produced by the VAA +Type so that comparisons could be made with putative pectinase-deficient mutants, isolated during this study. Furthermore the isozyme complement would give some indication as to the number of structural genes that code for PG and PL in VAA.

VAA produced PG at high levels on pectin and cell walls-salts media (Results and Discussion 1.1.1.; Cooper & Wood, 1975) but it was not induced on glucose and CMC on which only trace levels were detected, indicative of basal synthesis. As VAA may produce a range of isozymes in inducing media that are not produced during basal synthesis the PG and PL isozyme patterns were compared by flat bed and column IEF.

2.2.1. Flat bed IEF of polygalacturonase from pectin, cell walls, glucose and CMC cultures.

Filtrates from pectin, cell wall, glucose and CMC salts cultures (pH 5.0, MES 0.05 M) were concentrated by ammonium sulphate precipitation,

dialysed and subjected to flat bed IEF followed by activity staining for PG to determine any differences in the isozyme profiles produced in the four media.

A range of PG isozymes from pI 3-10 was synthesised in pectin and cell wall media with a particularly active isozyme of pI 6.9 (Plate 6). In contrast only a single isozyme was detected in filtrates from glucose and CMC cultures of pI 6.9.

2.2.2. Column IEF of polygalacturonase and pectin lyase produced by *V. albo-atrum*.

For preparation of PG and PL isozymes, concentrated pectin/salts culture filtrates were focused in a 110 ml LKB isoelectric focusing column (Materials and Methods 9.2.1.).

2.2.2.1. Broad range (pH 3-10) column IEF of polygalacturonase.

PG was induced in pectin/salts media (pH 5.0, MES 0.05 M) in which PL was recovered only at low levels (Results and Discussion 1.1.1.). Concen trated filtrate from 4 x 100 ml 7 d cultures was focused in a pH range of pH 3-10 (Materials and Methods 9.2.1.1. At least 100 RVU ml^{-1} PG activity was detected in all fractions from pH 3-10 (Fig 47); *c* 50 % of the activity was concentrated in the mid region at pI 6.9, coincidental with the highly active band found at this pI by flat bed IEF.

2.2.2.2. Broad range (pH 3-10) column IEF of pectin lyase.

Many attempts were made to resolve and detect pectin lyase by flat bed IEF including presoaking in 0.01 M $CaCl_2$ and an overlay of pectin (pH 8.0, 0.05 M). Unfortunately the substrate did not adhere to the PAG to allow sufficient ruthenium red staining. Attempts were made therefore to incorporate the substrate into the gel by mixing pectin (0.2 % w/v)

Plate 6. Isozyme profiles of polygalacturonase produced by *V. albo-atrum* on pectin (1), CMC (2), tomato cell walls (3) and glucose (4).

Plate 7. Polygalacturonase isozyme profiles of *V. dahliae* isolates.

(1) 20B; (2) TS 1; (3) TS 2A; (4) TS 2A; (5) 105; (6) RG.

Plate 6

Plate 7

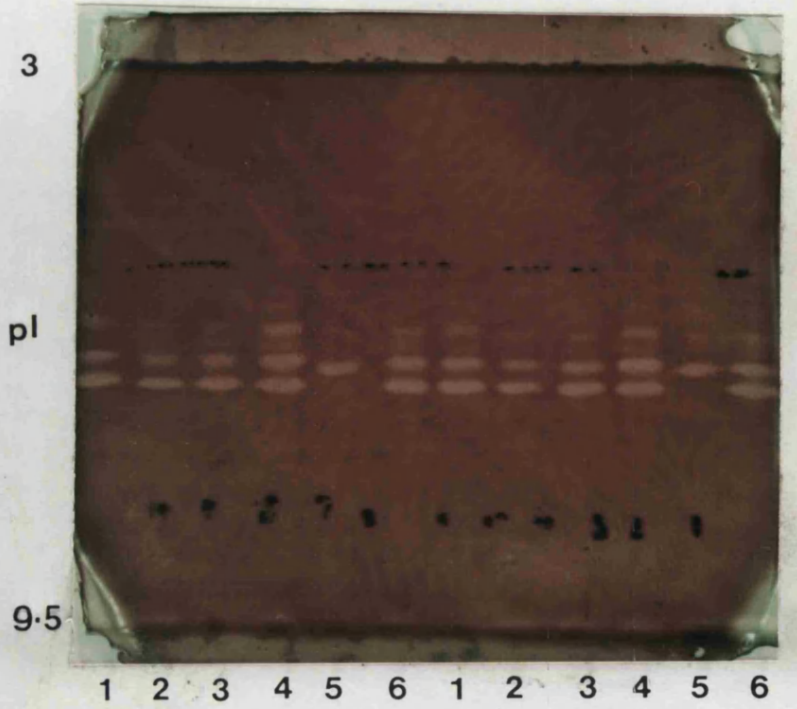


Fig 47. Broad range isoelectric focusing (pH 3-10) of polygalacturonase from *V. albo-atrum* pectin/salts cultures. PG (O), protein (—), pH (--).

Concentrated pectin/salts culture filtrate (pH 5.0, MES 0.05 M) was fractionated on an LKB 8101 IEF column (110 ml) in a pH gradient 3-10.

Total protein applied to column: 5.89 mg.

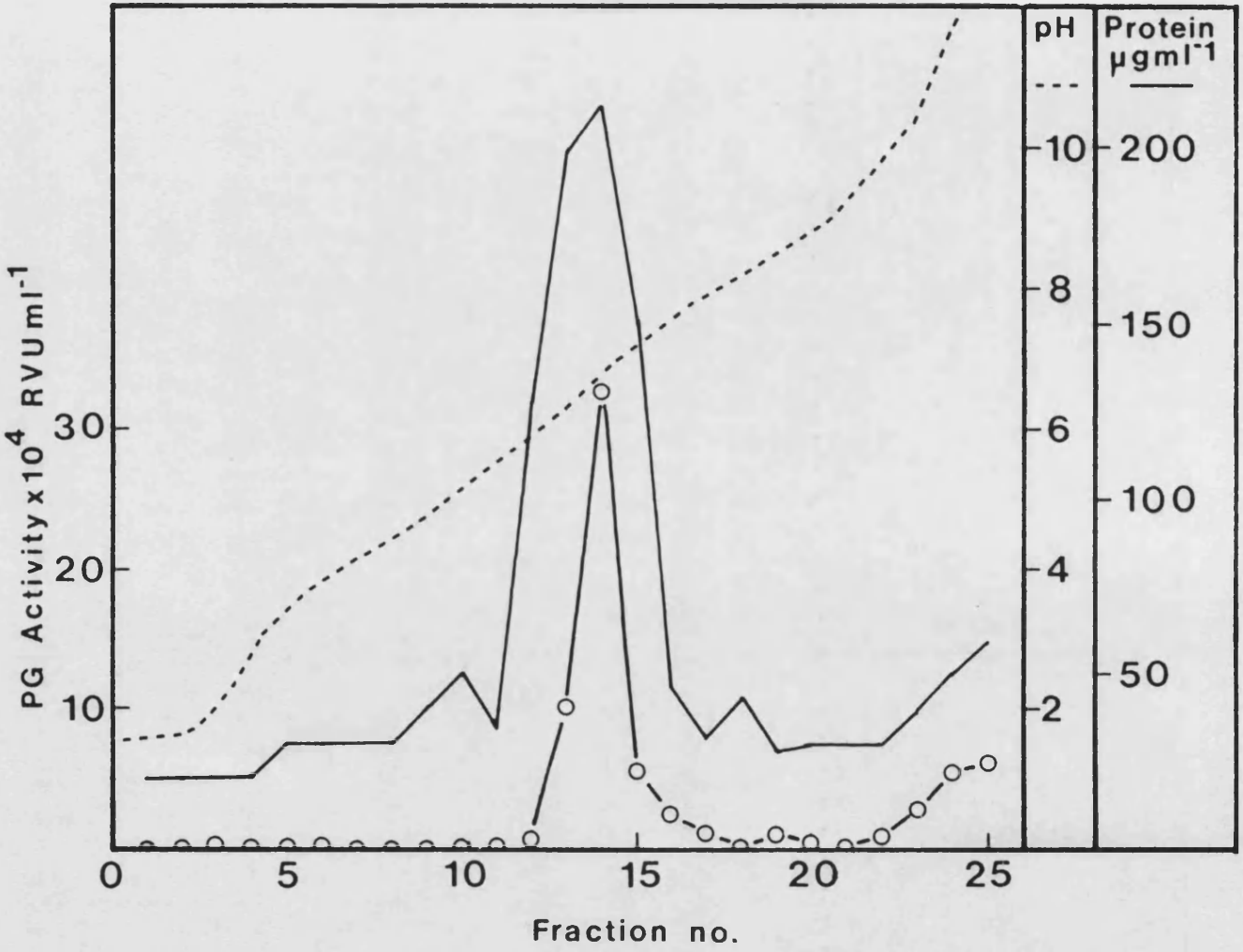
Total PG activity applied to column: 834 900.

Fraction size: 4.4 ml.

Maximum PG activity, fraction 15: 34 487 RVU ml^{-1}.

Representative results of three replicate experiments.

Fig 47


pH
Protein µgml⁻¹
PG Activity x10⁴ RVU ml⁻¹
30
20
10
10
8
6
4
2
200
150
100
50
0
5
10
15
20
25
Fraction no.

with buffered agarose (2 % w/v; pH 8.0, HEPES, 0.1 M) containing $CaCl_2$ (0.1 M) after first autoclaving the two components (116°C, 10 min); a 0.5 mm overlay of this mixture was prepared on Gel-Bond support film, applied to the focused gel and weighted with glass sheets. After 60 min incubation the gels were separated and stained with ruthenium red. Unfortunately this procedure, adapted from Andro *et al.* (1984) failed to reveal the PL isozymes. PL isozymes were therefore resolved by column IEF.

PL was induced in alkaline pectin/salts media (pH 8.0, HEPES 0.05 M) in which PG was recovered only at very low levels (Results and Discussion 1.1.1.). Concentrated filtrate from 3 x 100 ml 7 d cultures was focused as described above.

5 peaks of activity were detected by broad range IEF (Fig 48). The PL isozyme profile from alkaline pectin and cell wall culture filtrate were identical; the isozymes induced on both media are therefore the same. The highest peak was in the alkaline region ranging from pH 9-11. There was no detectable PL in alkaline glucose and CMC media and no isozymes were found in concentrated culture filtrate after IEF.

2.2.2.3. Narrow range (8-10.5) column IEF of pectin lyase.

Fractions 8.5-11 were further fractionated by narrow range IEF on a pH gradient of 8-10.5. Two distinct peaks were obtained confirming the presence of at least two isozymes with pI's 9.0 and 10.7 (Fig 49).

2.2.3. Determination of molecular weight and Stokes' radius of *V. albo-atrum* polygalacturonase and pectin lyase by gel-filtration.

Concentrated culture filtrates, rich in PG or PL activity were eluted through a Sephadex G-100 chromatography column as described in Materials and Methods 10.

Fig 48. Broad range isoelectric focusing (pH 3-10) of pectin lyase from *V. albo-atrum* pectin/salts cultures. PL (●), protein (—), pH (--).

Concentrated pectin/salts culture filtrate (pH 8.0, HEPES 0.05 M) was fractionated on an LKB 8101 IEF column (110 ml) in a pH gradient 3-10.

Total protein applied to column: 2.48 mg.

Total PL activity applied to column: 78 000 $\mu g\ ml^{-1}\ h^{-1}$.

Fraction size: 4.4 ml.

Maximum PL activity, fraction 22: 312 $\mu g\ ml^{-1}\ h^{-1}\ ml^{-1}$.

Representative results of three replicate experiments.

Fig 48

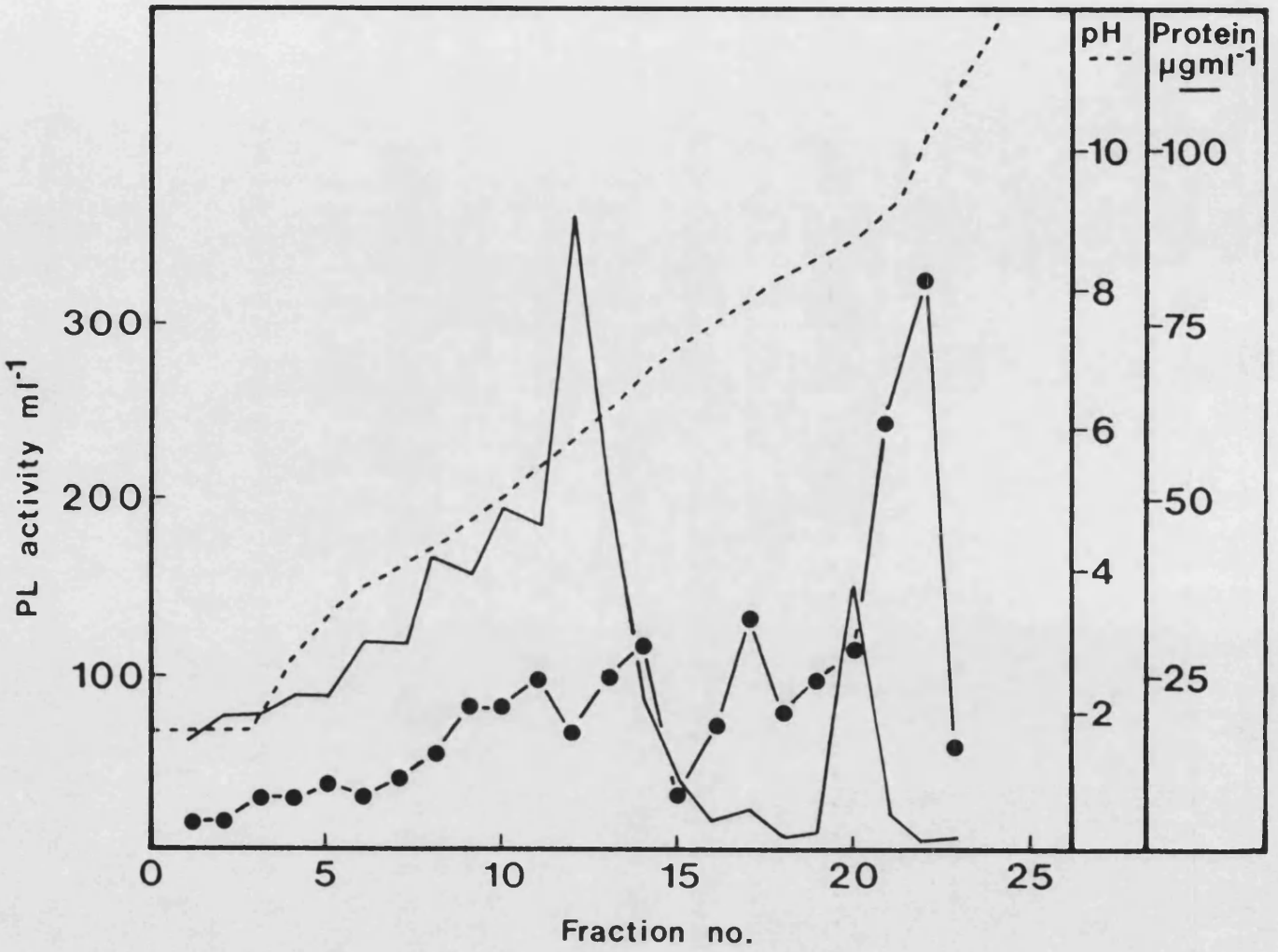

pH
Protein µgml-1
PL activity ml-1
300
200
100
10
8
6
4
2
100
75
50
25
0
5
10
15
20
25
Fraction no.

Fig 49. Narrow range isoelectric focusing (pH 8.5-11.0) of pectin lyase of *V. albo-atrum*. PL (●), protein (—), pH (- -).

Fractions pH 8.5-11.0 from broad range IEF (pH 3-10) were dialysed and fractionated on an LKB 8101 IEF column (110 ml) in pH gradient 8.0-10.5.

Total protein applied to column: 0.42 mg.

Total PL activity applied to column: 2 100 $\mu g\ ml^{-1}\ h^{-1}$.

Fraction size: 4.4 ml.

Maximum PL activity, fraction 4: 59 $\mu g\ ml^{-1}\ h^{-1}\ ml^{-1}$.

Representative results of three replicate experiments.

Fig 49

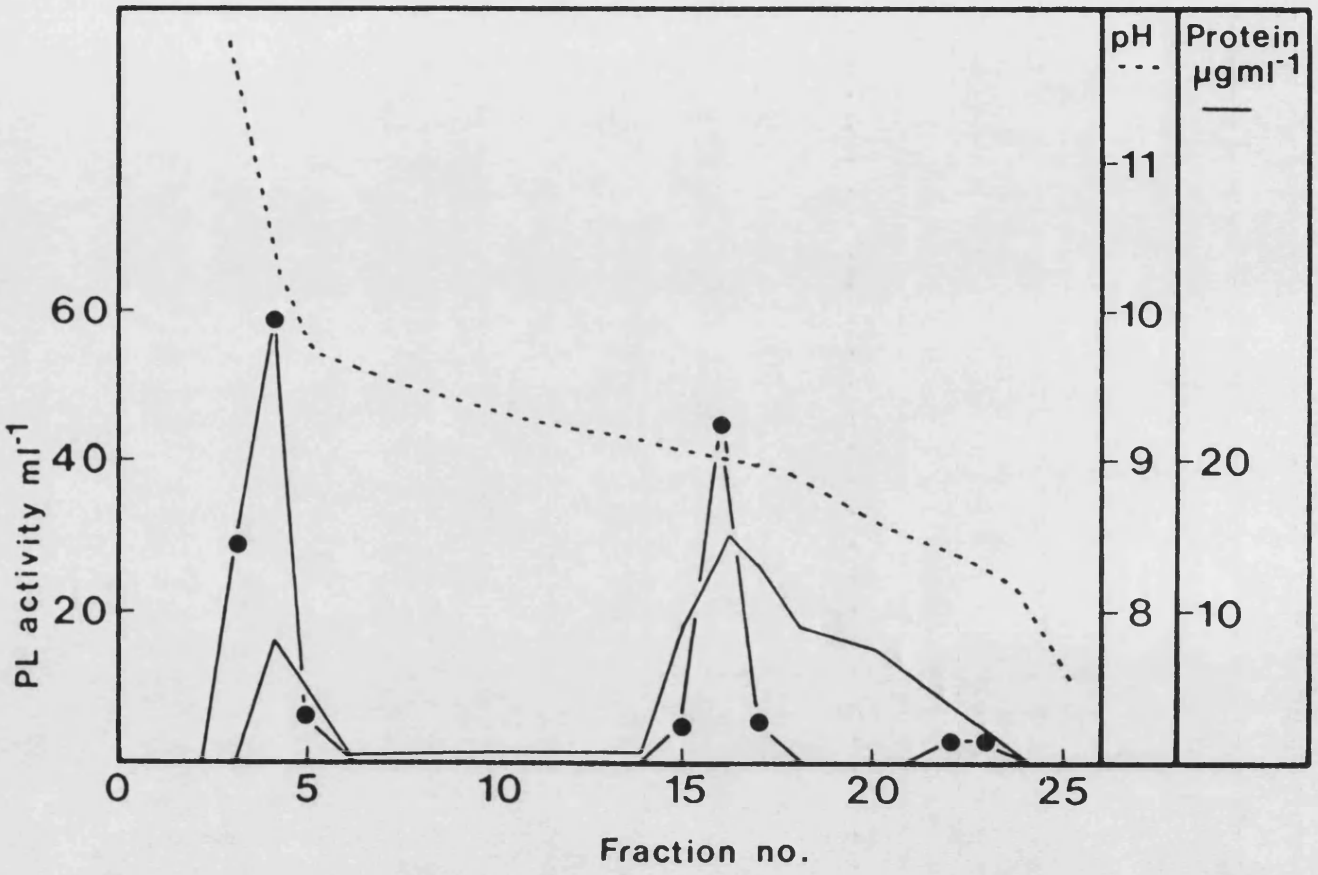

pH
Protein µgml⁻¹
PL activity ml⁻¹
60
40
20
11
10
9
8
20
10
0
5
10
15
20
25
Fraction no.

Fig 50. Sephadex chromatography of *V. albo-atrum* polygalacturonase.

Concentrated pectin/salts culture (pH 5.0, MES 0.05 M) filtrate was eluted down a column of Sephadex G-100. PG (O), protein (—).

Column length: 47 cm.

Column diameter: 2.6 cm.

V_t: 249.5 ml.

V_o: 87 ml.

Elution rate: 5 ml h^{-1}.

Fraction size: 4.6 ml.

Total protein applied to column: 0.92 mg

Total PG activity applied to column: 11 200 RVU.

Maximum PG activity: 250 RVU ml^{-1} fraction.

Results representative of three elutions

Fig 50

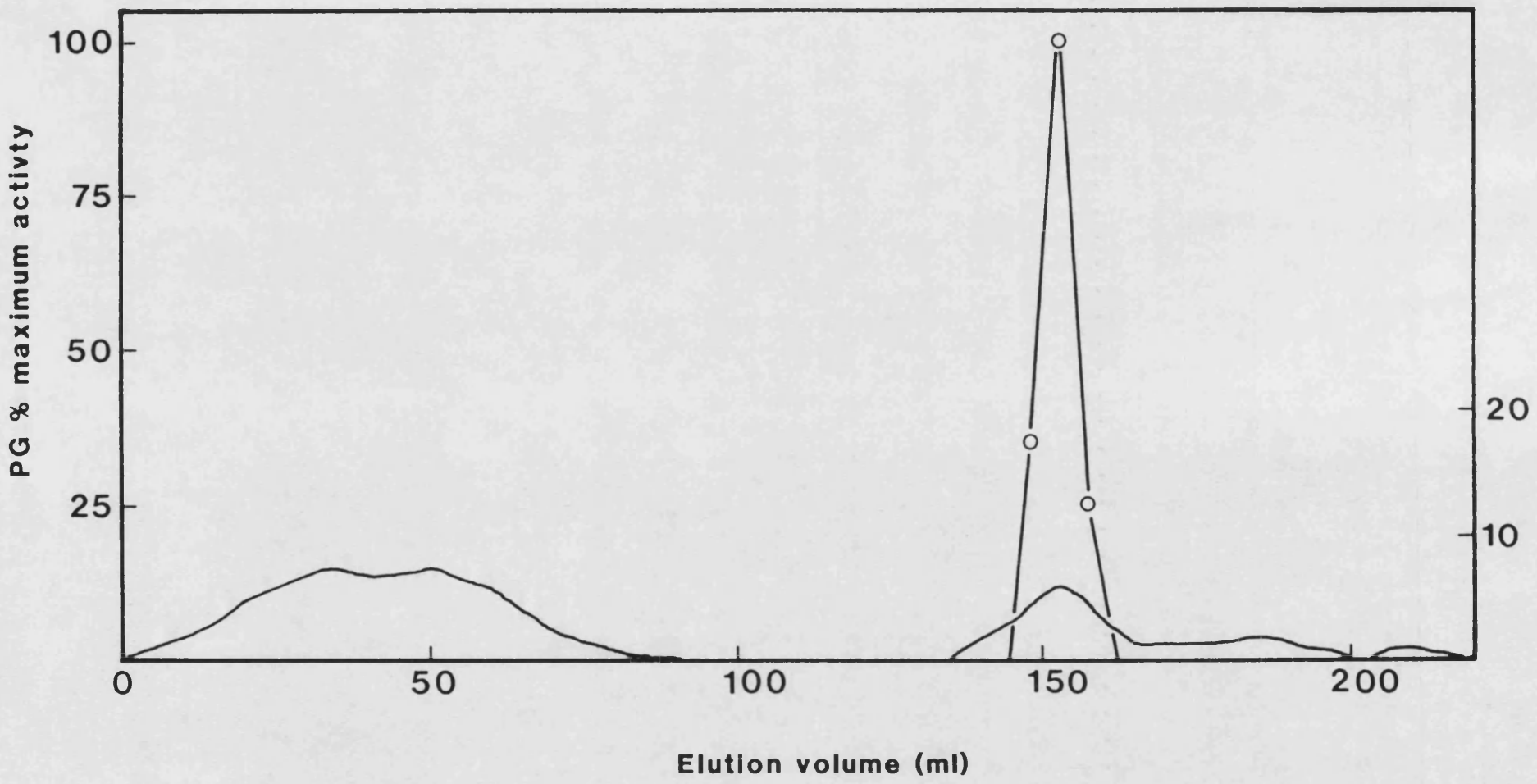

PG % maximum activty
100
75
50
25
0
50
100
150
200
20
10
Elution volume (ml)

Fig 51. Sephadex chromatography of *V. albo-atrum* pectin lyase.

Concentrated pectin/salts culture (pH 8.0, HEPES 0.05 M) filtrate was eluted down a column of Sephadex G-100. PL (●), protein (—).

Column length: 47 cm.

Column diameter: 2.6 cm.

V_t: 249.5 ml.

V_o: 87 ml.

Elution rate: 5 ml h^{-1}

Fraction size: 4.6 ml.

Total protein applied to column: 1.35 mg

Total PL activity applied to column: 31 200 μg ml^{-1} h^{-1}.

Maximum PL activity: 218 μg ml^{-1} h^{-1} ml^{-1} fraction.

Results representative of three elutions.

Fig 51

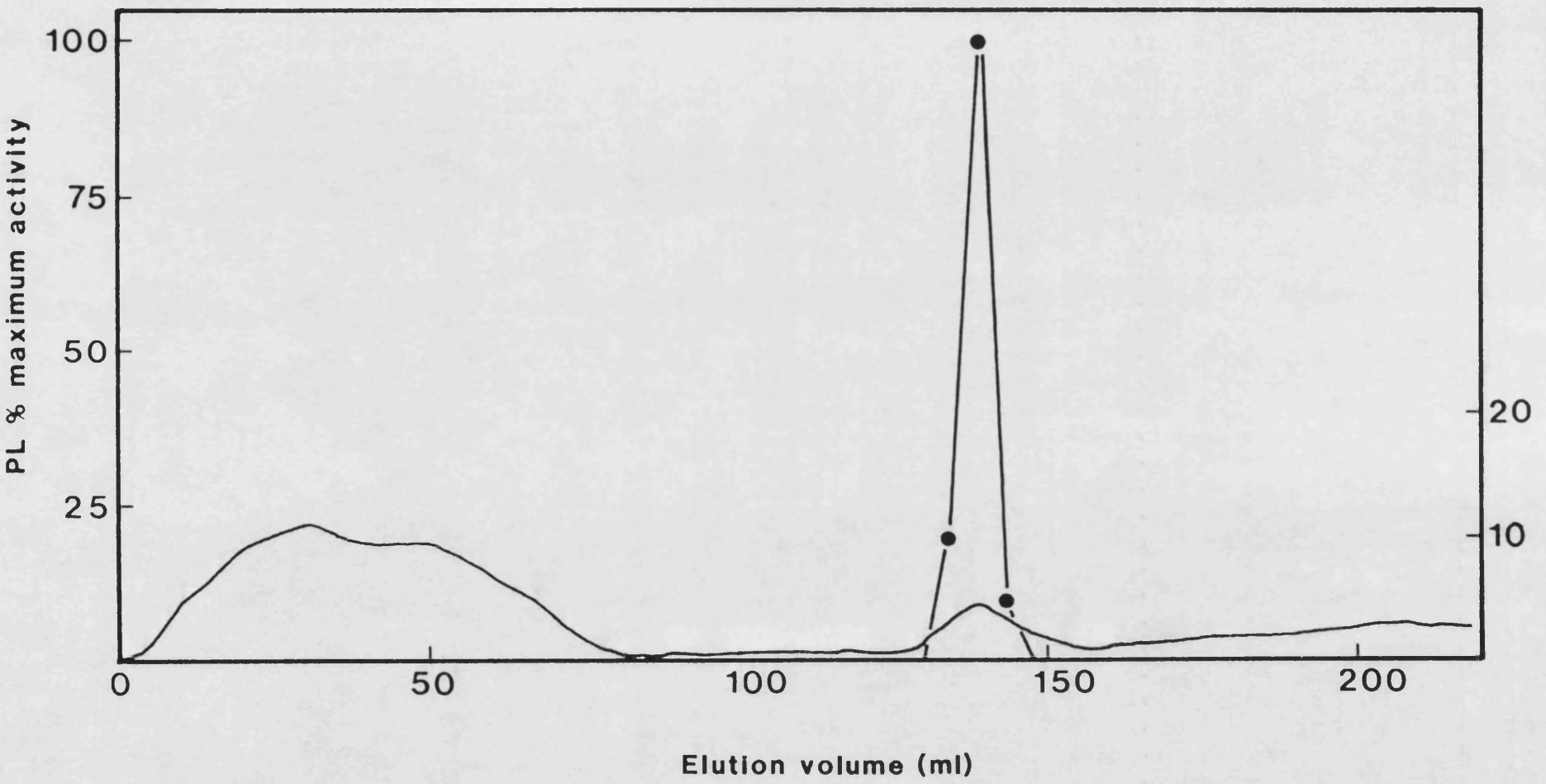

100
75
50
25
PL % maximum activity
0
50
100
150
200
Elution volume (ml)
20
10

Table 16. Molecular weights and Stokes' radii of *V. albo-atrum* PG and PL as determined by gel-filtration (see Figs 50 and 51). *a*

	Molecular weight (daltons)	Stokes' radius *b* (Å)
Sample	PG	PL
PG from conc. pectin/ salts culture filtrate (pH 5.0)	33 500	32.1
PL from conc. pectin/ salts culture filtrate (pH 8.0)	50 000	27.0

a Column diameter 2.6 ml, elution rate 4.6 ml h^{-1}, fraction size 4.6 ml
b Molecular weights and Stokes' radii were estimated from elution constants (K_{av}) of marker proteins and peaks of PG and PL activity as described in Materials and Methods 10.

PG and PL have respective MW of 33 500 and 50 000 daltons forming distinct peaks with K_{av}'s of 0.408 and 0.323, (Figs 50 and 51; Table 16).

2.3. Polygalacturonase isozyme profiles of +Type and auxotrophic *V. dahliae* isolates as determined by flat bed IEF.

V. dahliae is the second major *Verticillium* species of economic importance to crop production. This close relative of *V. albo-atrum* is also a vascular parasite and produces PG, PL and PME (Talboys & Busch, 1970), but no attempt has been made to establish whether these enzymes are produced in multiple forms. Furthermore, no correlation has been found between pathogenicity of *V. albo-atrum* isolates and isozyme patterns produced *in vitro* (Mohan & Ride, 1984) or in overall pectinase synthesis of Verticillium species (Talboys & Busch, 1970).

Resistance of tomato to VAA and *V. dahliae* is conferred by the presence of a single gene (Ve) which was introduced from a wilt tolerant Peruvian Wild strain of tomato (Schaible, 1951). However, new race 2 strains have appeared in the Mediterranean and Australia which are pathogenic

to tomato plants resistant to race 1 (J. Clarkson, pers. comm.).

Isozyme markers are now frequently used in studies of the dynamics and statics of gene populations (Burdon & Marshall, 1983) and it would be of interest to determine possible differences between the PG isozyme patterns produced by the two *V. dahliae* races. Furthermore there may be similarities between isolates originating from adjacent regions. Also an examination of PG multiplicity would indicate the potential of *V. dahliae* as a suitable candidate for obtaining PG-deficient mutants.

2.3.1. Polygalacturonase isozyme profiles of *V. dahliae* isolates races 1 and 2 from different geographical origins.

The isolates listed in Table 17 were grown in pectin/salts culture (pH 5.0, MES 0.05 M) to induce PG. After 7 d incubation on a rotary incubator (25°C, 150 rpm) the culture fluids were filtered from the mycelium and concentrated by ammonium sulphate precipitation overnight at 4°C. Two separate samples of concentrates (diluted to 5 ml volume) from two independent 100 ml cultures of each isolate were resolved by broad range (pH 3-10) flat bed IEF and activity stained with NAPP as outlined in Materials and Methods 9.2.2.2.1.

The 11 isolates were resolved into 3 groups according to 3 distinct PG isozyme banding patterns (Plates 7, 8 and 9; Table 17). There was no correlation between pathotype and pattern. There was however close similarity between the Australian isolates, T34-Arg from Greece and all of the N. American isolates, except 105. These isolates produced 3 major isozymes (pI's 6.2, 6.6 and 6.8) and at least 2 minor bands. Two of the Greek isolates, Egp and T64 had 2 minor isozymes and 2 of the major ones but distinctly lacked isozyme pI 6.8. 105 from N. Carolina had the simplest banding pattern possessing only one major isozyme (pI 6.7) which was common to all of the strains tested, and also 2 minor bands were present.

Table 17. Race, geographic origin, number and PG isozyme pattern group of *V. dahliae* tomato isolates (see Plates 7, 8 and 9). (*a*)

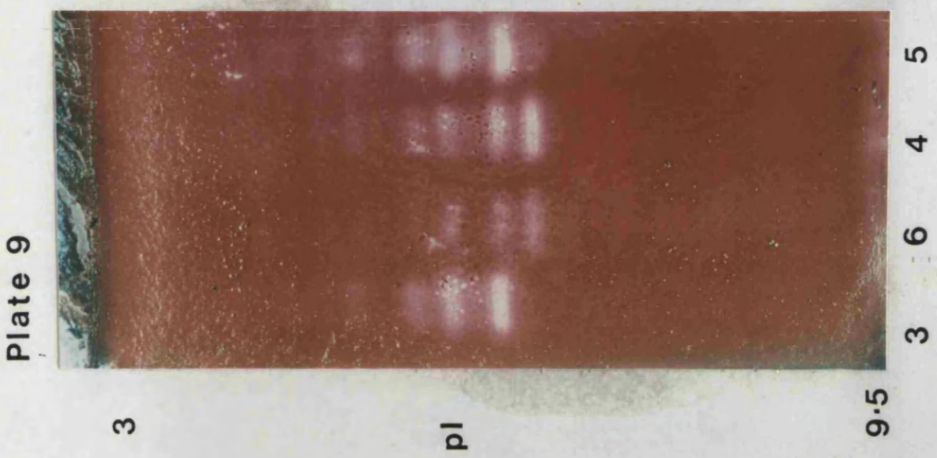
Isolate	Race		Origin	Source	PG Isozymes	
					No.	pattern group (*c*)
TS-1	1		California, USA	1	5	1
TS-2A	2	(*a*)	California, USA	1	5	1
TS-2B	2	(*a*)	California, USA	1	5	1
20B	1		N. Carolina, USA	2	5	1
105	2		N. Carolina, USA	2	3	2
RG	2		N. Carolina, USA	2	5	1
Egp (*b*)	2		Greece	3	4	3
T34-Arg	1		Greece	3	5	1
T64	1		Greece	3	4	3
T58	1		Australia	4	5	1
77-10C	1		Australia	4	5	1

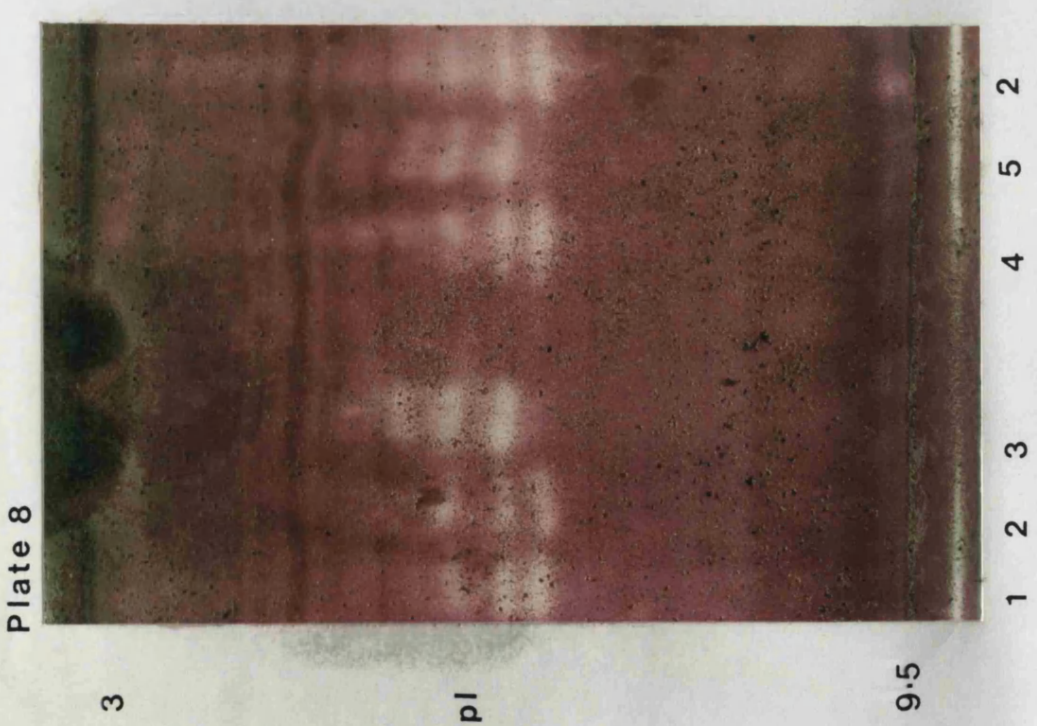
a TS-2a is a hyaline mutant of the melanised +Type TS-2B.
b Egp race 2 was isolated from eggplant.
c Three distinct PG isozyme patterns were identified. Each isolate could therefore be assigned to a group.

Source of isolates: 1) W.J.Tolmsoff, National Research Laboratory, Texas, USA; 2) R.G. Gardener, Mountain Horticultural Crops Research Station, N. Carolina, USA; 3) E.C. Tjamos, Phytopathological Institute, Athens, Greece; isolates 4) R.G. O'Brien, Department of Primary Industries, Indooroopilly, Australia.

Plates 8 and 9. Polygalacturonase isozyme profiles of *V. dahliae* isolates.

(1) RG; (2) 7710C; (3) Egp; (4) T58; (5) T64; (6) T34 Arg.



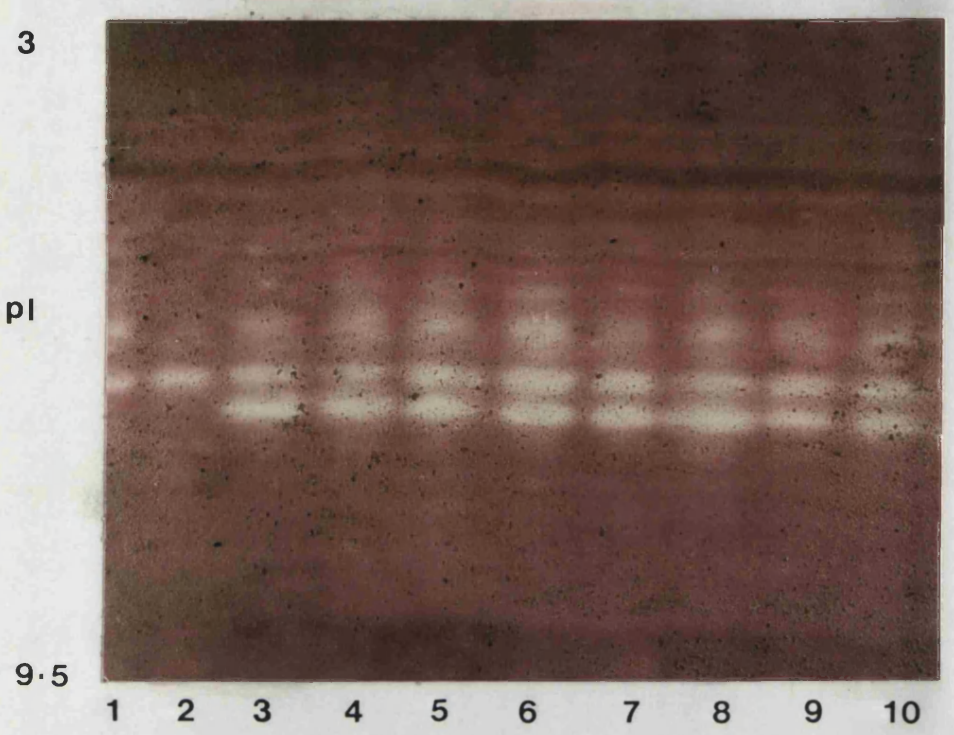


2.3.2. Polygalacturonase isozyme profiles of the parents and progeny of a parasexual cross between *V. dahliae* auxotrophs RG and 105.

The results in Table 17 show that two of the N. American isolates, 105 and RG, had very different PG banding patterns. As the PG isozymes may be useful markers in parasexual analysis and auxotrophic strains had previously been derived from the +Type isolates, the PG isozyme profiles of parents and prototrophic progeny were investigated. Alterations in pattern from the parents would indicate recombination during haploidisation in the parasexual cycle and could possibly provide information on linkage between the PG markers.

An auxotroph of 105 requiring arginine (105-arg) and a nicotinamide requiring RG (RG-nic) were isolated after NTG mutagenesis by Clarkson (1984). From a forced parasexual cross of the two auxotrophs (Hastie & Heale, 1984; Clarkson & Heale, 1985b), six progeny (P1, P2, P3, P4, P6 and P10) were obtained. These, in addition to the parents and the +Type RG and 105 isolates were grown in pectin/salts media as described above. The auxotrophs grew relatively poorly in this minimal media as vitamins were not added to avoid CR, however sufficient PG was induced for subsequent analysis. Dialysed concentrated culture filtrate was resolved by flat bed IEF and the PG was detected by activity staining. Both auxotrophs retained the same banding pattern as their respective +Types (Plate 10). All six progeny showed a PG isozyme profile typical of RG and unfortunately no recombinants were obtained.

Plate 10



Polygalacturonase isozyme profiles of parental *V. dahliae* auxotrophs and progeny of a parasexual cross.

(1) isolate 105; (2) auxotrophic parent 105 Arg (auxotrophic for arginine); (3) isolate RG; (4) auxotrophic parent RG Nic (auxotrophic for niacin); (5, 6, 7, 8, 9 and 10) respectively progeny P_1, P_2, P_3, P_4, P_5 and P_{10}.
+Type isolates and derivative auxotrophs have identical PG profiles. All progeny have PG isozyme profiles which closely resemble the RG parental isolate. There was no indication of recombination of PG isozyme genes from the PG isozyme profiles of the progeny.

2.4. Discussion of the properties of polygalacturonase and pectin lyase produced by *B. allii*, *V. albo-atrum* and *V. dahliae*.

The major PG isozyme (pI 5.4) produced by *B. allii* isolate 2070 principally degrades its substrate by random cleavage, as determined by its mode of action against NAPP where rapid viscosity reduction was accompanied by a comparatively slow rate of substrate hydrolysis (Table 12 and Fig 41). During initial hydrolysis this isozyme achieved a 50 % decrease in viscosity when < 0.16 % of the bonds had been cleaved and thus, by definition, is an *endo*-enzyme (Nasuno & Starr, 1967). However, later on, there was a sharp increase in GALA release although the accompanying drop in viscosity was only very slight. This suggests that PG isozyme pI (5.4) is capable of multiple attack. The increase in hydrolysis may have been in response to a rise in availability of amenable substrate; polypectate chains released by the action of the *endo*-acting active site were consequently amenable to cleavage by an *exo*-acting active site on the PG isozyme. The action pattern of this *B. allii* PG isozyme contrasts with PGL's of *E. carotovora* where the rate of cleavage declined as substrate chain length decreased (Moran *et al.*, 1968). It also contrasts with that of the *endo*-PGL of *Bacillus* species RK9, where a typical exponential reduction in viscosity was accompanied by a steady rate of NAPP-lysis (Kelly & Fogarty, 1978). The action pattern of *B. allii* PG isozyme pI 5.4 resembles more closely that followed by the PG's of *V. albo-atrum* and *F. oxysporum f. sp. lycopersici* and PGL of *X. campestris* (Cooper *et al.*, 1978; Nasuno & Starr, 1967). Large intermediate breakdown products, initially released, were subsequently cleaved to di and tri-GALA and UGALA.

The action of the complete PG activity from pectin/salts culture filtrate suggests the additional presence of *exo*-acting enzymes,

because viscosity reduction of NAPP was accompanied by relatively rapid substrate hydrolysis during all the stages of incubation (Fig 41). This action pattern resembled more closely that of a PG of *B. cinerea*, partially purified by gel filtration which reduced the viscosity of a 1 % solution of NAPP when 4.6 % of the glycosidic bonds had been hydrolysed (Urbanek & Zalweska-Sobczak, 1975). The primary uronide released by this PG after 10, 20, 60 and 120 min was mono-GALA which implies that this relatively large enzyme (MW 67 000) or a component has a capacity to cleave the substrate chains terminally.

The main degradation product of the major *B. allii* PG isozyme and total PG activity was di-GALA. Most of the mono-GALA was released from cleavage of tri-GALA and not from terminal cleavage of the larger chains. Di-GALA was released during early stages of degradation but it was clearly the second product of tri-GALA breakdown. *B. allii* isozyme (pI 5.4) could not degrade di-GALA unlike the PG of *B. cinerea* (Hancock *et al*, 1964a). A component of the total PG activity was however capable of cleaving di-GALA into mono-GALA, as by 2 h incubation only the monomer was present in the reaction mixture. Similarly, leaf hydrolysates and extracts of onion infected with *B. allii* contained only the monomer (Hancock *et al.*, 1964a). Speculatively, *exo*-PG's could provide nutrients or inducers for further PG synthesis where the primary rôle of the *endo*-PG's is to kill host cells and initiate substrate degradation. By using a combination of viscometric and TBA assay techniques it should be possible to identify any *exo*-PG activity from the range of PG isozymes as these enzymes typically release GALA residues concurrent with a negligible reduction in viscosity.

Preliminary detection of highly active *exo*-PG's may be achieved by activity staining of IEF gels, in which they form faint clearing zones in the substrate overlay (Ried & Collmer, 1985). Some of the weaker

bands on the IEF plates (Plate 4), particularly in the alkaline region, suggest the presence of *exo*-PG's. Alternatively these are *endo*-PG isozymes produced in very low quantities.

The PG of *B. allii* shares a pH optimum similar to that of PG's produced by many pathogens including *B. cinerea* (pH 4-5) and VAA (pH 5.0) (Cooper *et al*, 1978; Verhoeff & Warren, 1972). As the pH of onion tissue decreased from 6.25 to *c* 4.5 in the lesion areas, during pathogenesis, this pH optimum would be suitable for wall degradation *in vivo*. Similarly the pH fell from 6.2-4.5 in lesions on sunflower caused by *S. sclerotiorum* which favoured PG activity (Hancock, 1966).

PG was capable of degrading pectin to some extent, presumably because the substrate was incompletely methylated. Similarly, the ability of *E. carotovora* PGL to degrade pectin was dependent on the degree of substrate demethylation (Moran, Nasuno & Starr, 1968). *B. allii* additionally produces PME (Hancock *et al.*, 1964a), and although not investigated during this study, removal of the methyl groups from the pectin by the action of this enzyme would presumably facilitate further degradation by the PG isozymes.

The major, acidic *B. allii* PG should not readily bind to the negatively charged carboxyls on the polypectate polymer. However, ionic binding would appear to be essential for substrate degradation by PG as activity was inhibited in the presence of ions that presumably interfered with the formation of enzyme-substrate bonds, which could occur on the carboxyl groups. Monovalent K^+ ions may have inhibited enzyme activity in this way, whereas in contrast, the results suggest that Cl^- ions had a direct effect on the enzyme. Increased concentrations of ions and particularly Ca^{2+} inhibited PG isozyme 5.4 and overall PG activity against NAPP. Ca^{2+}, because of its ability to

link up the polypectate chains, would have prevented ionic binding of the enzyme and additionally excluded it from the substrate lattice. Ca^{2+} content has been related to disease susceptibility (Bangreth, 1979; Verhoeff, 1974). Ca^{2+} content of onions declines with age (Zink, 1966) which coincides with the increase in susceptibility to *Fusarium oxysporum f. sp. cepi* (Holtz & Knox-Davies, 1985b); also onions grown in soils with a low Ca^{2+} content were much more susceptible. It has been suggested by Holtz & Knox-Davies that susceptibilty is related to *Fusarium* PL which is more active in the presence of Ca^{2+} although no evidence was provided in support of this theory. For *B. allii*, it could follow that older bulb tissue, with a low Ca^{2+} content is more susceptible to attack by the fungus and the action of its PG's. However, increased susceptibility to *B. allii* or *B. squamosa* was not correlated with bulb age (stored from 2-12 weeks prior to inoculation) (Stewart & Mansfield, 1985a).

B. allii PG and isozyme pI 5.4 killed onion tissue more rapidly than VAA PG killed tomato stem parenchyma, at similar activities (Cooper *et al.*, 1978). Furthermore there was a more significant increase in K^+ leakage from the onion tissue. This may reflect a greater destructive ability of the *B. allii endo*-PG or in contrast, the onion tissue, which consisted of single cell layers, was relatively more amenable to degradation. Although cell killing and maceration are linked, in the case of *B. allii* PG, the tissue was not detectably 'loosened' by the effects of the enzyme until 24 h incubation, at which time *c* 90 % of the host cells were already dead (Table 15 and Fig 45). This could indicate that 1) cell killing precedes maceration and the latter process is secondary to the degradative effects of PG; 2) wall integrity is not directly related to leakage and viability loss; 3) limited wall damage occurred during the early stages of degradation and

lead to cell killing indirectly eg, by the release of toxic wall fragments. Loss of electrolytes from potato tissue occurred 3 min after exposure to Ech PGL and preceded maceration and cell death by *c* 15 min (Bateman & Basham, 1975a). Cell death was more closely related to maceration than to ion loss (Basham & Bateman, 1976). Cooper *et al* (1978) even found that loss in cell viability occurred several hours after the tomato tissue had weakened as a result of PG exposure. In contrast, the results with *B. allii* PG /onion tissue more closely resembled the effects of PL from *M. fructigena* on viability of apple suspension cells, which was reduced concurrently with ion leakage (Hislop *et al.*, 1979). Furthermore, cell wall degradation appeared to accompany rather than precede severe ultrastructural damage (Keon, 1985).

As the major *endo*-PG isozyme (pI 5.4) was detected in diseased tissue at the time of symptom formation, and, as this enzyme induces death of onion epidermis *in vitro* it would seem to have a likely rôle in pathogenesis (Results and Discussion 1.2.4.). However, some isozymes produced *in vitro* may not be found in diseased tissue, eg, of three PGL isozymes of *E. atroseptica* only one was recovered from rotted potato potato tissue (Quantick, Cervone & Wood, 1983). It was concluded that differential appearance of PGL was attributable to its inactivation by chlorogenic acid released from disrupted host cells. In contrast, the absence of an isozyme(s) might reflect on the extraction procedure whereby less active isozymes are lost by dilution or bound to the host tissue. This may be the case with *B. allii*/onion extracts where only the most dominant PG isozyme was detected. In some cases additional isozymes are apparent in infected tissue. *B. cinerea* does, however, produce a novel form of PG in apple tissue (Di Lenna & Fielding, 1983).

Their appearance may reflect the presence of alternative regulatory mechanisms for enzyme production, and/or on specific inducers that are not found *in vitro* but only in host tissue (Scott & Fielding, 1985). Although different forms of isozymes do arise *in vitro* and *in vivo*, pI's often do not markedly differ and reflect modifications by host enzymes or products bound to the enzyme surface (Cooper, 1986b; Di Lenna & Fielding, 1983).

Exo-polygalacturonate lyases are produced by several phytopathogens (eg *E. chrysanthemi*; Colmer *et al.*, 1982). However, *exo*-pectin lyases similar to that produced by *B. allii* in pectin, cell wall culture and in onion tissue have only been reported once before. Heale & Gupta (1972) reported 4 components in VAA pectin and NAPP culture filtrates which had *exo*-PL activity. However, others have reported only the production of *endo*-PL by this organism (Cooper, *et al*, 1978). As the former workers failed to include Ca^{2+} ions in the assays, it is possible that *endo*-PL was largely inactive because it has a partial requirement for this ion (Cooper *et al*, 1978). As a result of this omission the *exo*-PL was found exclusively in the filtrates. It would be interesting to know whether VAA *exo*-PL has a Ca^{2+} requirement as with the *B. allii* lyase and in contrast to the majority of lyases (Cooper, 1983), is inhibited by the ion.

The *exo*-PL produced by *B. allii* specifically degrades pectin, whereas many lyases will also degrade unmethylated substrate, eg *endo*-PL produced by *F. oxysporum* and VAA (Cooper *et al.*, 1978). VAA *exo*-PL cleaved mono-UGALA from pectin where in contrast *B. allii* lyase also released di-UGALA from the substrate. The rôle of *exo*-PL remains a mystery as although it is produced *in vivo* in relatively large quantities it does not kill onion cells or cause maceration. Furthermore, it would would probably be incapable of causing the

extensive wall breakdown necessary for rapid penetration and colonisation of host tissue. The degradation products of the PL may hold the key to its rôle. Mono and di-UGALA are presumably not phytotoxic, as under the experimental conditions host cell death occurred neither in the presence of the enzyme nor its UGALA products (which were detected by TBA at the end of the incubation period). It remains, therefore, that the mono- and di-UGALA may either be important for fungal nutrition and/or in the regulation of PG/PL synthesis (Results and Discussion 1.5.). This could be deduced by measuring PG/PL induction in restricted *B. allii* cultures, slowly fed with unsaturated uronides. Unfortunately these compounds are not available commercially and would have to be produced by enzymolysis (Collmer & Bateman, 1982). As growth on mono-GALA was poor, perhaps the unsaturated sugars are more acceptable for assimilation and metabolism by *B. allii*. As suggested above, some of the *B. allii* PG's may also be *exo*-types and it follows that their rôle also remains uncertain. Likewise, the rôle of an *exo*-PG, produced in onion tissue by *F. oxysporum f. sp. cepae* which does not macerate host tissue *in vitro*, also remains unknown (Holtz & Knox-Davies, 1985a). Di-GALA and di-UGALA are both released by the action of *exo*-PG and basal PGL activity produced by Ech and both of these compounds mediate induction of PGL synthesis on D-galacturonan (Collmer & Bateman, 1982; Collmer *et al.*, 1982). It should be possible to investigate the importance of *B. allii exo*-PG in nutrition and pectinase regulation once the enzymes have been purified and the degradation products identified, as discussed for *exo*-PL.

Four PG isozymes were found in germination fluids of *B. allii* conidia, but PG was only detected on agar containing pectin (Kritzman *et al.*, 1981). Trace PL activity was detected in onion cell wall cultures by Mankarios & Friend (1980) but unfortunately the enzyme was not isolated or characterised.

After gel-filtration most PG activity was concentrated in a single fraction; which presumably consisted of more than one isozyme of similar MW. As these isozymes are of equal MW, differences in pI must be attributable to small changes in structure rather than large differences in polypeptide chains. Some PG activity was eluted in a later fraction corresponding to MW 38 000. This fraction is probably a separate isozyme(s) as the MW precludes the possibility of dimerisation of the smaller unit.

The comparatively low MW *c* 27 000 daltons of *B. allii* PL (Table 10) is characteristic of many rhamnogalacturonan-degrading enzymes secreted by plant pathogens, eg the PL's 1 and 2 of *Colletotrichum lindemuthianum* which have respective MW's of 28 500 and 27 000 (Cooper, 1983; Wijesundera, 1984). The low MW would allow diffusion of *B. allii* PG and PL through the wall matrix which excludes globular proteins of MW 60-100 000 (Carpita *et al.*, 1979; Knee *et al.*, 1975). Clearly, free movement of these enzymes through the tissue would be important in enabling cell killing to occur in advance of the parasite.

In contrast, VAA PL was relatively large (MW 50 000). However, judging from the exclusion limits it should freely diffuse through the cell wall. The MW of VAA PG (33 500) was more typical of other fungal PG's.

Analysis of PG isozymes (Plates 7, 8 and 9; Table 17) revealed a very close similarity between the East and West coast N. American isolates of *V. dahliae* which indicates a degree of close common ancestory. The overall PG profile is presumably of an ancient origin which has been maintained during evolution. Alternatively the isolates are derivatives of one recent ancestor that has been spread rapidly over the continent, possibly as a result of cultivation and transport.

The close similarity between the Australian isolates, the Greek T34-arg and the American strains might suggest a close link between the

importation and transmission *via* superficially infected seed (Hawksworth & Talboys, 1970). Alternatively, because the Australian and N. American isolates belong to two incompatibility groups (J. Clarkson, pers. comm.), the results suggest that all of the PG isozyme genes have been carefully conserved and indeed have an ancient origin. This is of course speculative, as before the relative degree of conservation can be determined for a particular gene (in this case incompatibility genes or PG isozyme genes), the relevant selection pressure must be quantified. In simple terms there may be a greater pressure towards speciation *via* incompatibility than for progressive changes to occur in the PG isozyme profile.

As there was a substantial selection pressure for a successful pathotype to emerge from race 1 populations, that was capable of overcoming the single race 1 resistance gene Ve, it seems likely that race 2 strains could probably appear independently in different regions. Although it is impossible to speculate as to the selection pressure for changes in the PG isozyme complement, it would seem more likely that this would be of secondary importance; judging from the PG patterns, there is no distinction to be made between the PG profiles of the two races. Similarly, progressive and fluctuating VAA hop isolates failed to show distinctive correlations in PG isozyme patterns (Mohan & Ride, 1984).

The question arises as to why the *Botrytis* species, *V. dahliae* and particularly VAA possess so many pectinase isozymes. The answer may be found by determining the relative virulence and adaptability of these pathogens toward a number of different hosts. The two vascular parasites have been isolated from many varied host genera including numerous crop plants, eg VAA can infect aubergine, hop, lucerne and tomato; *V. dahliae* infects cotton, stone fruits, strawberry and various

solanaceous crops. *V. dahliae* has also been isolated from 64 weed species belonging to 28 families (Thanassoulopoulos, Biris & Tjamos, 1981). A large complement of PG and PL isozymes may confer a greater adaptive advantage to these pathogens during colonisation and would thus, increase their chances of survival. In respect of the very large number of PG isozymes which VAA produces this pathogen might be expected to have an even greater host range than *V. dahliae*. In contrast the *Botrytis* species are essentially host-specific (Stewart & Mansfield, 1985a). Perhaps the differences in their PG profiles preclude extensive cross infection of their respective hosts. Of 4 species of *Monilinia*, *M. laxa* which had the narrowest host, tissue and geographical ranges also had the least number of CWDE isozymes (Willetts, Byrde, Fielding & Wong, 1977).

It can be further postulated that the combined collection of isozymes includes 'specialist' enzymes and 'general' enzymes for host and non-host invasion and also, for saprotrophy. Different profiles may even confer a specialisation towards different tissue as, although *B. allii* and *B. squamosa* are both onion pathogens, they have exclusive PG profiles (Plate 3). The former is more infective towards bulb tissue, whilst the latter is more virulent on leaves (Stewart & Mansfield, 1985a).

The PG isozymes of *Botrytis* species, *B. allii* isolates, VAA and the *V. dahliae* isolates provide a ready source of distinctive characters for biosystematic analysis. Similarly isozymes of α-L-arabinofuranosidase, resolved by broad range column IEF, have been used to distinguish between species of *Monilinia* (Willetts *et al.*, 1977). PG and PL patterns were however conserved between the four *Monilinia* species examined and were thus of little taxonomic use. Isozyme patterns produced on flat bed IEF gels are invariably more complex than those

obtained by column IEF, and are consequently of more taxonomic value because of the greater number of characters. IEF is also a more powerful technique for producing enzyme characters than gel electrophoresis, eg many more enzymes were resolved for the *Botrytis* isolates by flat bed IEF than by Cruickshank (1983b) who used a pectin gel-electrophoresis technique (Cruickshank & Wade, 1980).

It was hoped that amongst the six progeny from the parasexual cross between *V. dahliae* isolates RG and 105, at least one recombinant would be found which possessed PG isozymes originating from genes of both parents. This was expected to be seen as an intense continuous band of activity between isozymes 6.6 and 6.8, or alternatively as a loss of one or more bands. If further crosses are forced between these auxotrophic strains a greater number of progeny will be available for screening, and this should increase the chances of isolating a recombinant. Recombinants of this kind could provide the basis of isozyme mapping in *V. dahliae*.

In the previous sections there is speculation on the relation between some properties of pectinases, PG/PL production *in vivo* and the rôle of the enzymes in pathogenesis. The next section reports on the attempts at resolving the importance of PG and PL *in vivo* by way of a genetical approach.

3. Isolation and testing of mutants *in vitro and in vivo.*

After establishing that the pathogenic isolates of VAA and *B. allii* produce PG and PL, which are regulated by induction and CR, and that the spores of both species are predominantly uninucleate and thus suitable for mutagenesis (Appendix 11), attempts were made to isolate mutants that were deficient in these enzymes. Such mutants would be of prime importance in establishing a direct correlation between PG and/or PL production with pathogenicity (Cooper, 1986) and would furthermore improve our knowledge of the mechanisms of pectinase regulation. This section reports on the approaches used to induce, select and characterise the mutants *in vitro* and *in vivo.*

3.1. Screening media and the selection of putative pectinase-mutants.

The media which have previously been used for the induction and detection of PG or PL were based on NAPP or pectin and unbuffered agar salts (Hankin & Anagnostakis, 1975; Howell, 1976). PG/PL activity was detected by flooding plates with the cationic detergent CTAB, which precipitates undegraded polymer to reveal cleared zones surrounding enzyme-producing fungal colonies. However, the pH of these media tended to drift markedly during incubation and were established to be non-specific for induction and detection of PG and PL (summarised in Materials and Methods 13). Therefore, to maximise the probability of obtaining specific PG^- and PL^- mutants, solid selection media had to be devised for efficient screening of colonies after mutagen treatment of conidia (Materials and Methods 13).

3.1.1. Mutual exclusiveness of PG and PL selection media.

As both PG^- and PL^- mutants were required, two selection media were devised which would both induce and detect the production of PG and PL

to the mutual exclusion of the other enzyme. Extracts of VAA colonies and agar in the surrounding enzyme cleared zones were tested for PG and PL activity to confirm the usefulness of the two media.

The acidic NAPP medium (N5) excluded PL activity and specifically selected for PG (Table 18; Plate 11). Conversely alkaline pectin medium (P8) excluded PG to 10 % of the activity around colonies on N5 medium but induced high levels of PL. PL was not detected on Czapek-Dox medium but PG was detected at very low levels, indicative of basal production (*c* 1 % of induced levels).

Table 19 shows the typical colony and clearing zone sizes attained by VAA on PG and PL detection media. PG clearing zones in NAPP always extended much further from the leading edge of colonies than those resulting from PL activity in the alkaline pectin medium. Although PL activity extended for only *c* 1 mm from the leading edge of hyphae it was readily distinguishable, on addition of CTAB, because the agar containing the colony was cleared against the grey background of undegraded pectin (Plate 13).

Table 18. Mutual exclusiveness of PG and PL selection media.

Media	Relative activity of VAA PG and PL around colonies *a*	
	PG *b*	PL *c*
Sodium polypectate 0.5 %, Ca^{2+} free, pH 5.0	100	0
Pectin 0.5 %, $+Ca^{2+}$, pH 8.0	11	100
Czapek-Dox, sucrose 3 %, pH 6.5	1	0

a Enzymes extracted in d.H_2O (10 ml) from 10 g agar/colonies and dialysed overnight at 4°C.
b PG activity determined viscometrically in NAPP (1 % w/v, pH 5.0); maximum activity 50 RVU g^{-1} agar/colonies.
c PL activity determined by TBA; maximum activity 118 $\mu g\ ml^{-1}\ h^{-1}$ UGALA released g^{-1} agar/colonies from pectin (0.5 % w/v, pH 9.0).

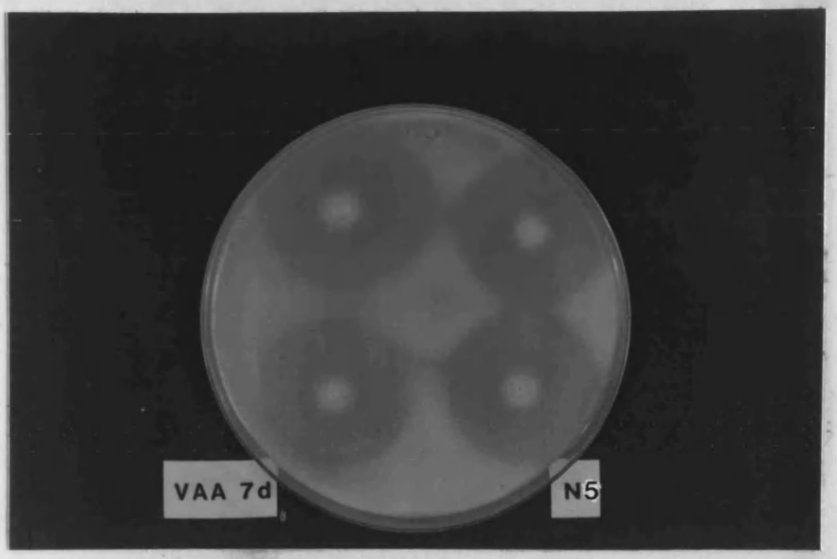
B. allii and *B. fabae* also produced large clearing zones on PG detection medium. *C. lindemuthianum* failed to produce clearing zones on PG medium, which is interesting as this PG is hyphal bound (O'Connell *et al.*, 1986); it did, however, produce an extracellular *endo*-PL and accordingly caused clearing zones in the PL detection medium. Clearing zones produced by *B. allii* on alkaline pectin medium were insignificant, and presumably due to either *exo*-PL activity or to weak *endo*-PG activity; although enzymes were not assayed in extracts. pH drift was minimal and remained very close to the original pH of the buffers (pH 5.0, citrate 0.05 M; pH 8.0, HEPES 0.05 M) in N5 and P8 media respectively (and in the presence of glucose; see below), in and around the colonies over the incubation period. As citrate did not cause CR in liquid media (Appendix 12), it was judged to be suitable for use in the solid selection medium; HEPES was unavailable as a carbon source, and therefore could not effect CR (Results and Discussion 1.5).

3.1.2. Selection media for the detection of de-repressed mutants.

Derepressed mutants may provide an insight into PG and PL regulation by CR, eg a mutant that was simultaneously de-repressed for PG and PL would strongly suggest the presence of an overall repression system for synthesis of both enzymes.

It was hoped that de-repressed mutants would be detected in the PG and PL inducing media by adding excess glucose, at 2 %, because enzyme production by +Type colonies should be repressed and remain at basal levels as occurs in liquid culture; Results and Discussion 1.1.1.), non-repressed mutants would be detected by the significant clearing zones produced around their colonies. However, clearing zones were formed around +Type colonies on both N5 and P8 media, in the presence of glucose (compare Plates 12 and 14 with 11 and 13). Therefore, to test the possibilities that clearing zones may have been produced;

Plate11



V. albo-atrum colonies and polygalacturonase clearing zones on N5 media, 7 d after inoculation.

Plate 12

V. albo-atrum colonies and polygalacturonase clearing zones on NG5 media, 7 d after inoculation.

Plate 13

V. albo-atrum colonies and pectin lyase clearing zones on P8 media, 7 d after inoculation.

Plate 14

V. albo-atrum colonies and pectin lyase clearing zones on PG8 media, 7 d after inoculation.

1) as a result of basal synthesis or 2) by induced synthesis following rapid depletion of glucose, determination of PG and PL, glucose, GALA and UGALA levels were made in agar around colonies on inducing medium and on inducing medium containing glucose.

VAA colonies grew 7-fold better on NAPP with glucose than on glucose alone (Table 20). PG activity, around colonies on NG5 medium, was also greater than around those grown on N5 medium (Table 21). However, in relation to the relative mycelial dry weights (from Table 19), the PG activity mg^{-1} colony dry weight (Table 20) is marginally greater on N5 medium. In other words PG activity is not entirely related to growth; the higher activity in N5 was due to induced synthesis whereas PG activity on NG5 medium, although high, was most likely to have originated from basal synthesis, as the glucose levels remained high and CR would thus have been operative (Table 22).

Table 19. Colony and enzyme zone diameters, fresh and dry weights of colonies in selection medium.

Media	Colony diam. (mm)	Enzyme degrad. zone diam. (mm)	Weight of colony and degradation zone (mg)	
			Fresh	Dry
a	*b*	*c*		*d*
N5	7	20	850	3.8
NG5	9	25	2730	26.8
P8	14	16	404	5.3
PG8	14	16	710	15.9

Mean results of three experiments.

a N5 medium: sodium polypectate (0.5 %, pH 5.0, citrate 0.05 M); NG5 medium: N5 medium and glucose 2 % w/v; P8 medium, pectin (0.5 %, pH 8.0, HEPES, 0.05 M); PG8 medium, P8 medium and glucose 2 %.
b VAA point inoculated onto the agar surface; incubated for 7 d, 23°C.
c CTAB added to replicated plates to develop areas of substrate degradation around colonies.
d Dry weights of colonies were determined by steaming the agar medium from the colonies on preweighed Whatman #1 filter paper, they were then dried to a constant dry weight at 60°C.

The differences in PL production are clearer between P8 and PG8 selection medium than for PG in N5 and NG5 (Table 20). Although VAA grew 3-fold more on pectin with glucose (measured by individual colony dry weight) than on pectin alone, the total activity in the extract of colonies and agar in PG8 (5 g fresh weight) was *c* 3 fold less (Table 21). When the relative mycelial dry weights are also accounted for, the difference is more pronounced; PL levels mg^{-1} were 4 x higher in the absence of glucose as, presumably, the glucose represses PL synthesis. PL produced in PG8 medium must therefore be synthesised basally, as the glucose levels remained at 1 130 $\mu g\ g^{-1}$ colony and agar (fresh weight; Table 22) which should be sufficiently high to cause CR.

Levels of GALA and UGALA residues were very high around the colonies of VAA grown on all the selection media. Levels were especially high, (> 1 000 $\mu\ g^{-1}$ colony/agar fresh weight from a possible 3 900 $\mu g\ g^{-1}$), on N5 and P8 medium (Table 22). 791.2 $\mu g\ g^{-1}$ GALA was found in extracts from around colonies on NG5 medium containing glucose, (which remained

Table 20. PG activity in N5 and NG5 selection medium related to mycelial mass. *b*

Media	Total PG in extract (RVU) *a*	PG (RVU) mg^{-1} mycelium	
		Fresh wt *b*	Dry wt *b*
N5	153	0.029	7.6
NG5	460	0.092	5.7

Mean results of three experiments.

a 5g colonies and the surrounding clearing zones was homogenised in a chilled mortar with 10 ml K phosphate buffer (pH 7.0, 0.1 M) and acid washed sand. The extract was filtered through Whatman # 1 paper. Aliquots of the extract (*c* 5 ml) were either dialysed overnight for enzyme assay or reserved for assaying uronide and glucose levels.
b Fresh weight includes agar. Dry weights determined as detailed in Table 19.

at 1 470 μg g^{-1}, but GALA was lowest on PG8 medium (133.5 μg g^{-1}) UGALA) although glucose levels had fallen from 20 000 μg g^{-1} to 1 130 μg ml^{-1}. Growth on P8 was 1.5 fold greater than on N5, which, probably explains why uronic acid levels were lower on the former medium, as the degradation products presumaby would have been assimilated and metabolised more rapidly.

The uronide degradation products from N5 and P8 media were analysed by TLC (Plate 15). Mono-GALA alone was found in extracts from N5 medium, whilst mono- and di-UGALA were found in P8 media. Free uronides were not found in extracts taken from uninoculated media.

The very low PL activity detected in PG8 (Table 21) may explain why the levels of UGALA were so low in extracts of this medium. Uronic acid levels were *c* 5 fold higher in N5 medium than in P8 which possibly reflects the relatively greater PG activity.

The high uronic acid levels around the colonies complicate the outcome of pectinase synthesis as these degradation products may themselves cause CR. In which case, although both PG and PL are initially induced on N5 and P8 respectively, and are not produced constitutively (but only at very low basal levels, on Czapek-Dox; Table 18), further synthesis of these enzymes may be repressed by the presence of excess free uronic acid in the surrounding medium.

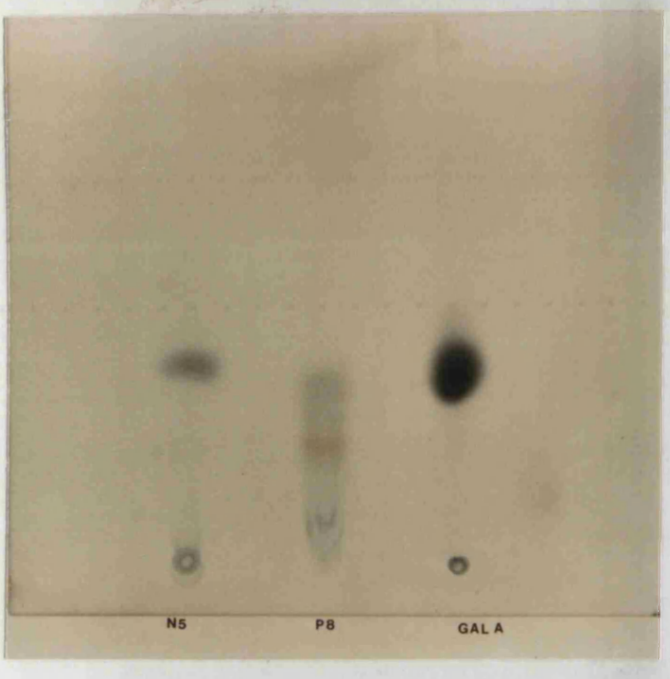
Table 21. PL activity in P8 and PG8 selection media related to mycelial mass. *a*

Media	Total PL (μg ml^{-1} h^{-1}) in extract	PL (μg ml^{-1} h^{-1}) mg^{-1} mycelium	
		Fresh wt	Dry wt
P8	873	0.16	13.4
PG8	245	0.085	3.3

Mean results from 3 experiments.

a See Table 3 for experimental details.

Plate 15



Thin-layer chromatograms of NAPP and pectin degradation products extracted from around colonies of *V. albo-atrum* grown for 7 d on N5 and P8 selection media.

Mono-GALA was used as a standard.

From the results in Table 23 it is clear that that PL activity is prone to inhibition by a dialysable factor present in the P8 and PG8 extracts. It is likely that the enzyme is subject to end-product inhibition, as levels of UGALA were quite high in the medium. When UGALA was removed by dialysis, PL activity increased markedly (by > 10 x). In contrast, PG activity decreased following dialysis; PG was less vulnerable to end-product inhibition by GALA whereas activity was halved by dialysis (Table 23). The susceptibility of the lyase to inhibition by UGALA may explain why the clearing zones were so small around the VAA colonies growing on this medium (Plate 13).

Table 22. Galacturonic acid, unsaturated galacturonic acid and glucose concentrations around colonies in selection medium.

	Sugar concentration (μg) in colony and agar g^{-1} fresh weight	
Media	GALA/UGALA *a*	Glucose *b*
N5	1 477	0
NG5	791	1 470
P8	1 105	0
PG8	133	1 130

Mean results from 3 experiments.

a GALA was released in acidic medium (N5 and NG5) from the degradation of NAPP by PG; UGALA released in alkaline medium (P8 and PG8) by PL, as determined by TBA. The maximum possible uronic acid concentration in N5 was 3 900 μg g^{-1}.
b Determined by glucose oxidase assay. Maximum glucose concentration (in control uninoculated agar) was 20 000 μg g^{-1}.

Table 23. Effect of dialysis on enzyme activity in extract ml^{-1}. *a*

Media	GALA/UGALA (μg) *b*	PG activity (RVU) c	PL activity (μg ml^{-1} h^{-1}) *b*
N5 undialysed	312	59	0
N5 dialysed	0	32	0
NG5 undialysed	159.5	200	0
NG5 dialysed	0	89	0
P8 undialysed	147	0	17
P8 dialysed	0	0	194
PG8 undialysed	22	0	32.5
PG8 dialysed	0	0	43

Mean results from three experiments.

a Samples dialysed overnight in 200 vol. d.H_2O at 4°C.
c Assayed viscometrically.
b Assayed by TBA.

3.1.3. Frequency of isolation of mutants on selection media.

Aliquots of spores were spread onto large 14 cm plates of selection medium at suitable dilutions so that *c* 100 colonies developed from the surviving spores following mutagenesis (Materials and Methods 12). Colonies that apparently lacked or had reduced clearing zones were noted and reisolated from the Czapek-Dox replicate plates. After further culture on new selection medium and on Czapek-Dox (to reduce the chance of losing them), most of these putative mutants were rejected as on rexamination they were clearly producing pectinases, either because they had reverted or because they had failed to grow on Czapek-Dox or minimal pectin selection medium and were therefore presumably auxotrophic. A record was made of the total number of colonies screened and of the number of mutants isolated of VAA and *B. allii* (Table 24).

Table 24. Total surviving colonies of *V. albo-atrum* and *B. allii* screened on polygalacturonase and pectin lyase detection medium for PG or PL deficiency, and the number of mutants obtained.

Mutant	*V. albo-atrum*		*B. allii*	
	Total screened	no. isolated	Total screened	no. isolated
PG⁻ *a*	20 000	0	30 000	0
PL⁻ *b*	30 000	3	0	0

a PG deficient mutants isolated on N5 selection medium for PG detection.
b PL deficient mutants isolated on P8 selection medium for PL detection.

Three VAA PL mutants were isolated which represented 0.01 % from 30 000 survivors screened on P8 medium. PG mutants of VAA were not isolated from N5, the PG detection medium. PG and PL hyper-productive mutants were not isolated.

However, of the putative mutants obtained from the three screens on P8 medium, three stable mutants were obtained which showed on further culture, on P8, a reduced capacity to produce PL. One strain (111b), which was initially thought to be hyper-productive on plates, was shown to be identical to +Type in later experiments. 111b was retained and used as a positive control in further experiments in which the +Type was compared with the 3 mutant strains. 111b, thus provided evidence that the selection procedure *per se* did not cause a loss in pathogenicity. The detailed characterisation of these 5 isolates *in vitro* and *in vivo* is reported in the following Sections.

B. allii produced discrete colonies with distinct clearing zones in the N5 selection medium. However, no *B. allii* PG⁻ mutants were obtained, although at least 30 000 colonies were screened, in three separate experiments. The mutagen was operative as *c* 90 % of the spores were killed, presumably from the effect of NTG mutagenesis (Materials and

Methods 12). No PG hyper-productive mutants of *B. allii* were isolated. Time precluded attempts at isolating pectinase-mutants of *C. lindemuthianum*, although this pathogen produces PL on P8 selection media and would therefore be a suitable candidate for the procedure (Literature Review 3.7.).

3.2. *In vitro* characterisation of putative mutants.

A direct correlation between altered pathogenicity and PG/PL production can only be established if the PG and PL-deficient mutants are dissimilar to the +Type exclusively in the characters that were selected for. Therefore, the production of a wider range of CWDE and other enzymes needed to be studied, in order to detect any pleiotropic effects resulting from point mutations in structural genes or genes controlling pectinase production.

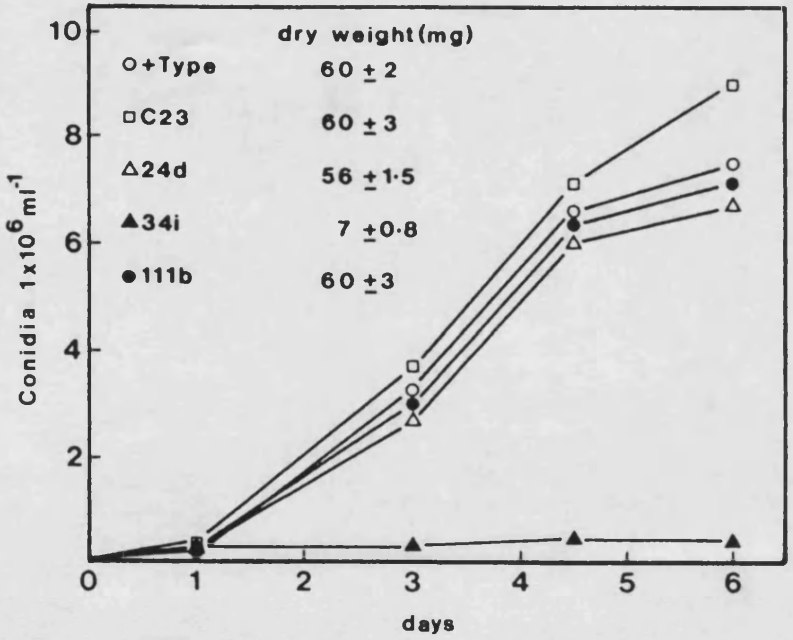
In addition, morphology and growth patterns were assessed because any discrepancies between the mutants and the +Type would suggest additional alterations in genes, indirectly related to pectinase production.

3.2.1. Confirmation of pectin lyase deficiency in liquid culture.

The size of the clearing zones produced by the mutants on PL detection medium gave a relative guide to the reduction in PL activity (Table 25). However, PL production by the five isolates was also assessed in alkaline inducing medium containing pectin (0.5 % w/v) to gauge, Mutants 24d, C23 and 34i, selected for their small clearing zones on P8 medium, produced PL at a rate of *c* 43, 9 and 3 % of the +Type respectively, over 6 d in liquid alkaline pectin medium (Fig 53) confirming that they were partially PL deficient (Table 25). Conidial production and final dry weights were similar between all isolates and the +Type with the exception of 34i which grew very sparsely (Fig 52 and Table 25). The poor growth shown by 34i may either be a consequence

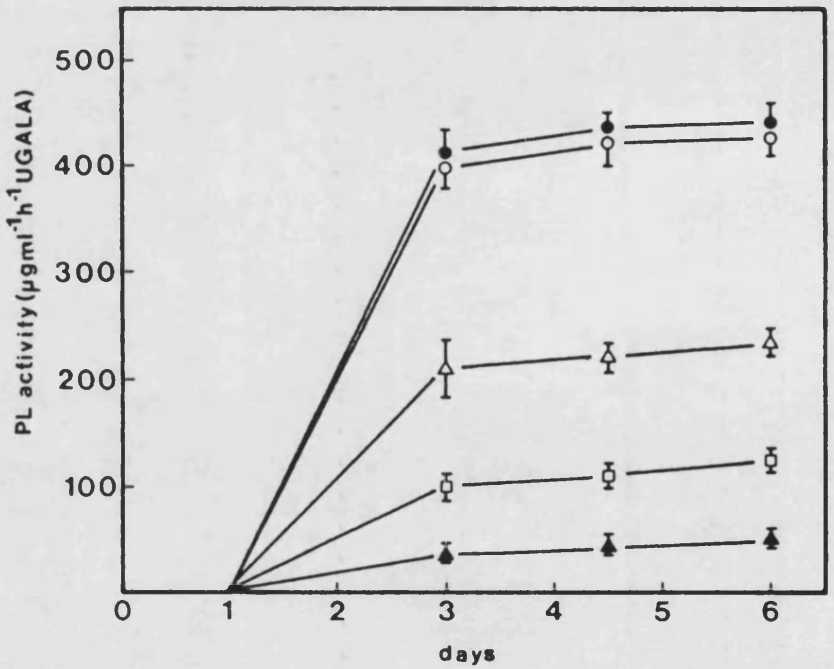
GROWTH AND PECTIN LYASE PRODUCTION BY V. ALBO-ATRUM +TYPE AND MUTANTS IN PECTIN SALTS MEDIA (0.5%, pH 8.0).

Fig 52



A. Conidial production and final mycelial dry weights. Mean results of 4 cultures; standard errors deleted for clarity.

Fig 53



B. PL production as determined by the release of UGALA from pectin (pH 9.0), measured by TBA.

Table 25. Colony growth and clearing zone diameters of +Type and PL⁻ mutant strains after 7 d incubation on N5 and P8 detection medium. *a*

Strain	N5		P8	
	colony diam. (mm)	clearing zone diam. (mm)	colony diam. (mm)	clearing zone diam. (mm)
+Type	11.1	23.2	14.1	16.4
C23	10.9	15.8	14.0	8.1
24d	11.5	22.1	13.9	10.2
34i *b*	4.1	4.1	3.5	3.5
111b	11.5	23.0	13.8	16.9

Mean results of 20 colonies.

a Plates of N5 (NAPP, 0.5 % w/v; pH 5.0) and P8 (pectin, 0.5 % w/v; pH 8.0) were point inoculated with spores from each isolate. The plates were incubated at 23°C. S.E. ≤ 0.2 mm for all colonies and clearing zones.
b The small clearing zones around 34i were also very faint.

or a cause of the low PL activity exhibited in the medium. 34i failed to utilise the UGALA degradation products, released from pectin by its minimal PL activity, as indicated by the high levels of UGALA that remained in the filtrates after 6 d (595 $\mu g\ ml^{-1}$); whereas the level of UGALA in cultures of the other isolates was considerably lower at *c* 190 $\mu g\ ml^{-1}$, presumably because much of the uronide had been assimilated and metabolised.

3.2.2. Production of pectinases and other cell wall degrading enzymes *in vitro*.

VAA +Type and mutant strains were grown on acidic pectin medium, mono-GALA, and tomato cell walls to determine the relative production of PG and a range of other enzymes, in addition to PL; growth on the different carbon sources was assessed.

Growth on acidic pectin salts medium was similar by all isolates with the exception of 34i which again grew very poorly (final mycelial dry weight 7 mg; Table 26). Growth on acidic pectin media was slightly better by all the other isolates at pH 5.0 than on alkaline pectin media; final dry weights of *c* 60 mg in alkaline conditions compared to *c* 75 mg in the acidic pectin medium (Fig 52). 34i again failed to utilise the free GALA residues, which remained in the culture fluids at 650 $\mu g\ ml^{-1}$ after 7 d whereas all of the hydrolysed pectin had been utilised by the other isolates (Table 26). These results in association with the previous findings (Results and Discussion 3.2.1.) suggest that 34i is a metabolic mutant which is incapable of metabolising (or perhaps assimilating) uronic acids.

The apparent inability of this mutant to grow on galacturonides was confirmed by inoculating cultures of mono-GALA with 34i (0.5 %, pH 6.5), in which it barely grew (final dry weight *c* 1 mg; Table 27) in contrast to the other isolates which attained *c* 60 mg. Neither PL nor

Table 26. Growth, PG and PL production by +Type and mutant strains on acidic pectin salts medium. *a*

Strain	PG (RVU) *b*	PL ($\mu g\ ml^{-1}\ h^{-1}$) *c*	dry weight (mg)	conidia ($10^6\ ml^{-1}$)	pH	GALA ($\mu g\ ml^{-1}$) *d*
+Type	500 ±25	41 ±3	75.2	1.88	6.8	0
C23	200 ±11	8 ±0.5	79.8	2.05	6.7	0
24d	470 ±10	22 ±1.5	70.7	1.84	6.7	0
34i	10 ±0.5	4 ±0.5	16.7	0.25	5.0	650
111b	522 ±20	43 ±4	81.0	1.80	6.7	0

Mean results of 4 replicate cultures.

a Pectin salts medium: 100 ml 0.5 % pectin at pH 5.0 (MES 0.05 M).
b PG assayed viscometrically.
c PL assayed by TBA.
d GALA concentration determined by TBA.

PG were produced by any isolate on GALA, even at detectable basal levels (Table 27). VAA pectinase synthesis is subject to CR (Cooper & Wood, 1975) and therefore PG and PL activities were not expected in these unrestricted cultures, in the presence of excess GALA.

PG production was favoured in acidic conditions and cultures of both +Type isolates and 24d contained *c* 500 RVU (Table 26). However, although C23 grew well on pectin/salts (pH 5.0) it only produced 40 % of +Type PG levels. 34i produced PG at *c* 1 % of +Type induced levels; subsequent evidence indicated that this much reduced production was basal synthesis (see Results and Discussion 3.2.4.).

All isolates were grown on tomato cell walls (0.2 %, pH 7.0, unbuffered) for 7 d and a range of enzyme activities were determined in the culture filtrate. Production of PL, PG on cell walls by VAA +Type and mutants resulted in similar differences in relative levels between the isolates, to those attained on alkaline and acidic pectin medium;

Table 27. Growth, PG and PL production by +Type and mutant strains in unbuffered GALA salts medium. *a*

Strain	PG (RVU) *b*	PL ($\mu g\ ml^{-1}\ h^{-1}$) *c*	dry weight (mg)	conidia ($10^6\ ml^{-1}$)	pH	GALA ($\mu g\ ml^{-1}$) *d*
+Type	0	0	52.0	0.52	8.9	920
C23	0	0	44.6	0.56	8.5	1 100
24d	0	0	47.2	0.43	8.9	830
34i	0	0	1.0	0.02	6.7	4 000
111b	0	0	54.0	0.54	8.8	840

Mean results of 4 replicate cultures.

a GALA medium: 100 ml 0.5 % mono-GALA initially adjusted to pH 6.5.
b PG assayed viscometrically.
c PL assayed by TBA.
d GALA concentration determined by TBA and absorbance at 515 nm.

however maximum levels were lower than those produced on pectin (Figs 54-57 and 53, Table 26).

Cellulase production was similar by mutant 24d, 111b and +Type. In contrast C23 produced very low levels of this enzyme and additionally reduced amounts of PG, PL, cellulase, β-D galactosidase, β-D glucosidase and L-leucine arylamidase were found; a correlation which strongly suggests that C23 is a secretory mutant (Table 28).

Anomalously 34i the apparent metabolic mutant produced *c* 1.6 fold more cellulase than the +Type, although growth, particularly initially, was comparatively poor (Fig 54); the low levels of the other enzymes produced by 34i may well reflect its poor growth on cell walls.

An overall semi-quantitative survey of enzyme activities employing the API-ZYM strips (Materials and Methods 6.6) showed that none of the mutants had lost any of the enzymes that the +Type produced in this medium (Table 29). C23 produced markedly lower levels of each enzyme which correlated with the reduction in PG, PL and cellulase activities (Table 28). The slow growing isolate 34i produced lower levels of some enzymes.

GROWTH, PL, PG AND CELLULASE PRODUCTION BY V. ALBO-ATRUM +TYPE AND MUTANTS IN MINIMAL SALTS MEDIUM CONTAINING TOMATO CELL WALLS (0.5 %). (Mean results of 4 cultures; standard errors deleted for clarity).

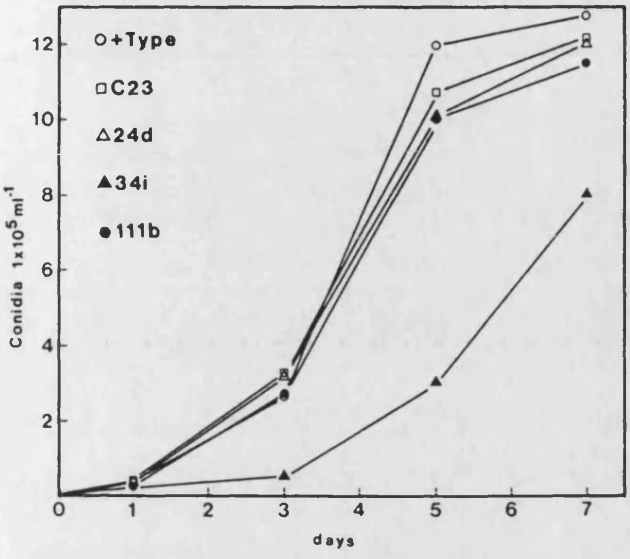


Fig 54. Conidial production.

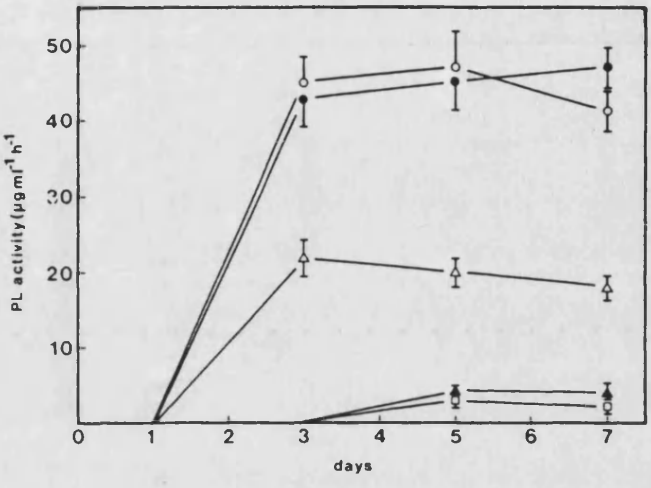


Fig 55. Pectin lyase production.

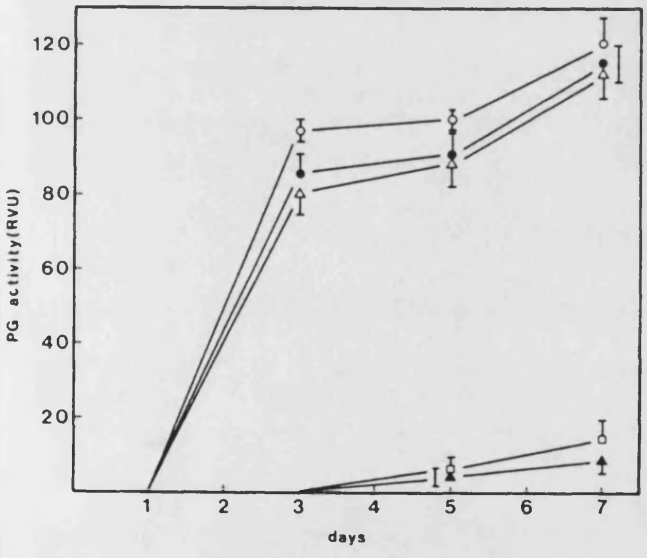


Fig 56. Polygalacturonase production.

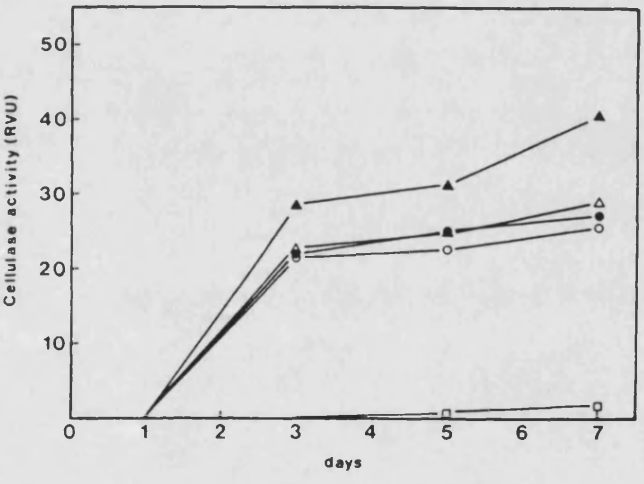


Fig 57. Cellulase production.

Table 28. RELATIVE ACTIVITIES OF ENZYMES PRODUCED BY V. ALBO-ATRUM +TYPE AND MUTANTS ON CELL WALLS (0.5 %, 7d)

STRAIN (Final pH)	PECTIN LYASE a	POLYGALACTURONASE b	CELLULASE c	β-D GALACTOSIDASE d	β-D GLUCOSIDASE e	L-LEUCINE ARYLAMINIDASE f
+TYPE (7.0)	100	100	100	100	100	100
C23 (6.7)	3	9	3	30	31	13
24d (6.8)	43.5	95	110	101	90	100
34ı (6.7)	9	7.5	164	34	30	7
111b (7.1)	113	98	105	115	92	90

a Determined by TBA. 100 % activity = 41 ug ml h ml enzyme$_1$
b Determined viscometrically. 100 % activity = 121 RVU ml^{-1} enzyme.
c Determined viscometrically. 100 % activity = 26 RVU ml^{-1} enzyme.
d Determined by activity against β-D galactopyranoside-PNP. 100 % activity = 0.12 u moles PNP h^{-1} ml^{-1} enzyme.
e Determined by activity against β-D glucopyranoside-PNP. 100 % activity = 0.1 u moles PNP h^{-1} ml^{-1} enzyme.
f Determined by activity against L-leucine-naphthylamide. 100 % activity = OD of 0.09 at 540 nm.

Table 29. Enzymes produced by +Type and mutants on tomato cell walls, as determined by API-ZYM.

	Enzyme activity in filtrate (0-5) (*a*, *b*)				
Enzyme	+Type	C23	24d	34i	111b
Alkaline phosphatase	0	0	0	0	0
Esterase (C_4)	4	2	4	2	4
Esterase, lipase (C_8)	2	1	2	1	2
Lipase (C_{14})	2	1	2	2	2
Leucine arylamidase	2	1	2	1	2
Valine arylamidase	2	1	2	1	2
Cysteine arylamidase	1	0	1	1	1
Trypsin	1	1	1	1	1
Chymotrypsin	0	0	0	0	0
Acid phosphatase	2	1	2	1	2
Phosphoamidase	1	0	1	1	1
α-galactosidase	1	1	1	1	1
β-galactosidase	1	1	1	1	1
β-glucuronidase	0	0	0	0	0
α-glucosidase	0	0	0	0	0
β-glucosidase	2	1	2	1	2
N-acetyl-β-glucosaminidase	1	1	1	1	1
α-mannosidase	0	0	0	0	0
α-fucosidase	0	0	0	0	0

Representative results of three replicate strips.

a Samples removed from dialysed culture filtrate.
b Enzyme activity in arbitrary units.

3.2.3. Determination of intracellular pectin lyase activity in putative secretory mutants.

If the PL$^-$ mutants were synthesising PL but unable to release it due to a genetically based disfunction in their secretory mechanisms, PL might be expected to accumulate within the cytoplasm. Intracellular PL was therefore determined within mycelium of each isolate, grown in alkaline pectin medium. Washed mycelia and conidia from 5 d pectin salts, (pH 8.0) cultures were homogenised, and subjected to ultrasonication (Materials and Methods 15.). α and β alkaline phosphatase served as

Table 30. Intracellular PL as a proportion of total activity.

Strain	Intracellular activity (% total) (*a*)		
	PL (*b*)	Alkaline phosphatase (*c*)	
		α	β
+Type	5.6	100	100
C23	23.8	100	100
24d	6.4	100	100
34i	(1.3) (*d*)	100	100
111b	6.0	100	100

Mean results of 3 experiments.

a Total activity: activity in 300 ml of dilaysed extracellular culture fluid and in 50 ml of dialysed mycelial/conidial extract. Intracellular extract prepared by homogenisation and ultrasonication over ice. Final dry weights of extracts (mg) as collected by centrifugation (17 000 x g) were; +Type, 35; C23, 35; 24d, 37; 34i, 7; 111b, 38.
b PL activity determined by TBA.
c 1 unit of enzyme activity is defined as the increase in absorbance of 1.0 of β-naphthlyamide released after 1 h incubation in reaction mixture. α-phosphatase activity; +Type, 1.2; C23, 1.2; 24d, 1.37; 34i, 0.04; 111b, 1.0. β-phosphatase activity; +Type, 1.38; C23, 1.24; 24d, 1.29; 34i, 0.15; 111b, 1.07.
d 34i grew poorly on pectin, thus PL activity extracted from only 7 mg extract.

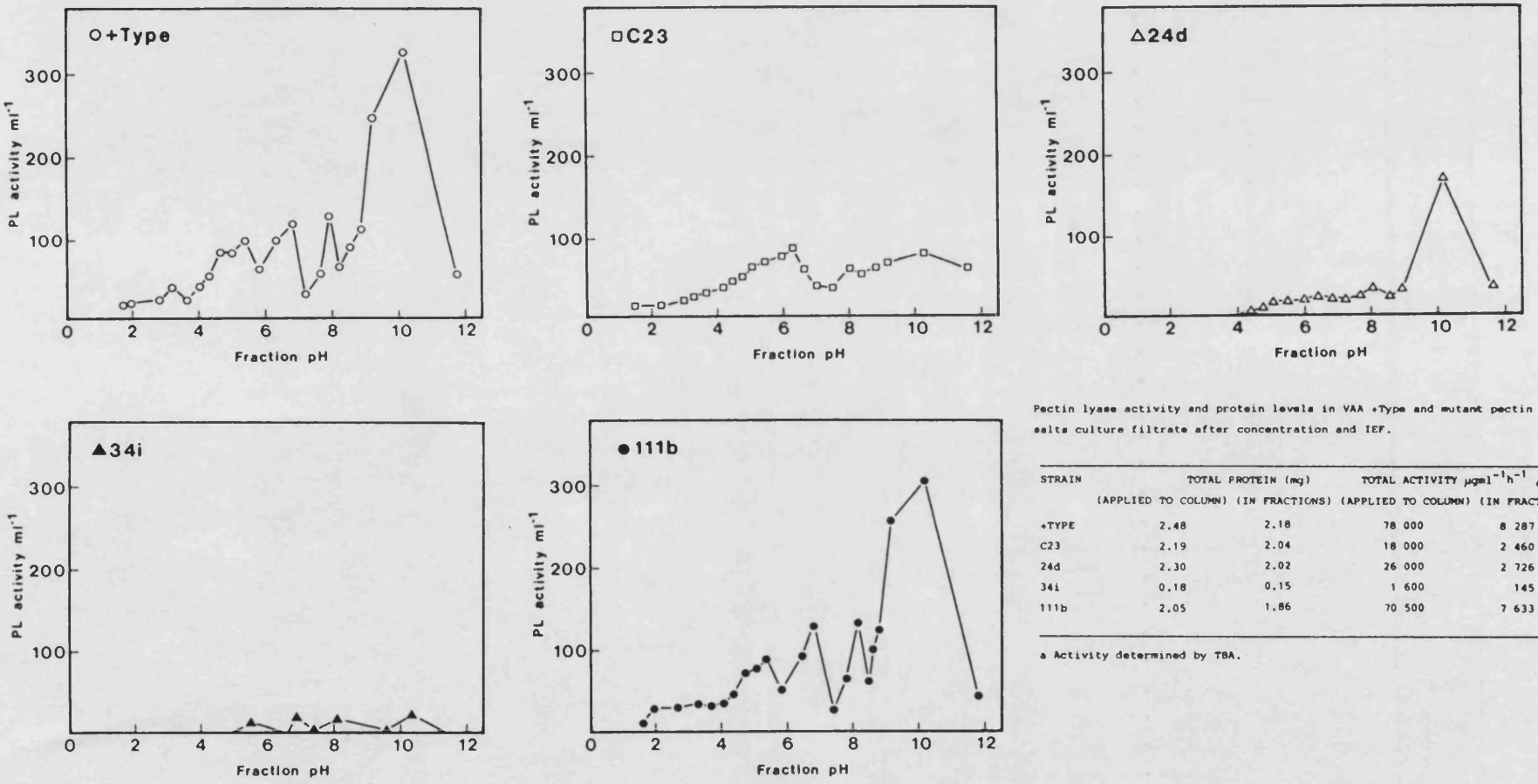
indicators of intracellular activity as phosphatases were not found in the extracellular fluids of 5 d cultures, but only appeared after prolonged incubation, when the mycelium was visibly autolysed. Intracellular PL constituted 5.5-6 % of the total lyase activity of +Type (and 111b), and 24d. However PL activity in comparable extracts of C23 mycelium and conidia was more than 4-fold higher, strongly suggesting a failure in this mutant's ability to secrete the enzyme into the medium. The extraction methods of homogenisation and ultrasonication did not significantly reduce PL activity in control solutions.

3.2.4. Variations in +Type and mutant polygalacturonase and pectin lyase isozyme profiles.

+Type VAA produces 6 PL isozymes and at least 20 PG isozymes (Results and Discussion 2.2.). The reduced PL and PG activity produced by the mutants could stem from the loss of one or more of the isozymes; alternatively, synthesis or release of all of the isozymes could be impaired. The role of individual isozymes or specific combinations in disease may be of greater importance than overall PG/PL activity, thus PL and PG isozyme profiles were determined for each mutant.

The PL profiles of the +Type and 111b, obtained by broad range IEF (3-10) in an LKB column were almost identical, confirming the existence of at least 6 isozymes (Fig 58.1 and 58.5). In contrast the profiles of C23 and 34i reflected an overall reduction in activity along the pH gradient. In particular both of these mutants produced proportionally low amounts of the two major alkaline PL isozymes (pI 9 & 10.6) (Fig 58.2 and 58.4). 24d produced the major PL isozyme (pI 10.6) at relatively high levels although the acidic PL isozymes and the second dominant alkaline isozyme (pI 9.0) were produced at very low levels.

Fig 58 ISOELECTRIC FOCUSING (pH 3-10) OF PECTIN LYASE FROM PECTIN SALTS CULTURES OF VAA +TYPE AND MUTANTS



Pectin lyase activity and protein levels in VAA +Type and mutant pectin salts culture filtrate after concentration and IEF.

STRAIN	TOTAL PROTEIN (mg) (APPLIED TO COLUMN)	TOTAL PROTEIN (mg) (IN FRACTIONS)	TOTAL ACTIVITY $\mu g ml^{-1} h^{-1}$ [a] (APPLIED TO COLUMN)	TOTAL ACTIVITY $\mu g ml^{-1} h^{-1}$ [a] (IN FRACTIONS)
+TYPE	2.48	2.18	78 000	8 287
C23	2.19	2.04	18 000	2 460
24d	2.30	2.02	26 000	2 726
34i	0.18	0.15	1 600	145
111b	2.05	1.86	70 500	7 633

a Activity determined by TBA.

Fig 59. Narrow range isoelectric focusing (pH 8.5-11.0) of pectin lyase of *V. albo-atrum* mutant 24d. PL (●), protein (—), pH (--).

Fractions pH 8.5-11.0 from broad range IEF (pH 3-10) were dialysed and fractionated on an LKB 8101 IEF column (110 ml) in pH gradient 8.0-10.5.

Total protein applied to column: 0.38 mg.

Total PL activity applied to column: 1 700 μg ml^{-1} h^{-1}.

Fraction size: 4.4 ml

Maximum PL activity, fraction 4: 57 μg ml^{-1} h^{-1} ml^{-1}.

Representative of three separate experiments.

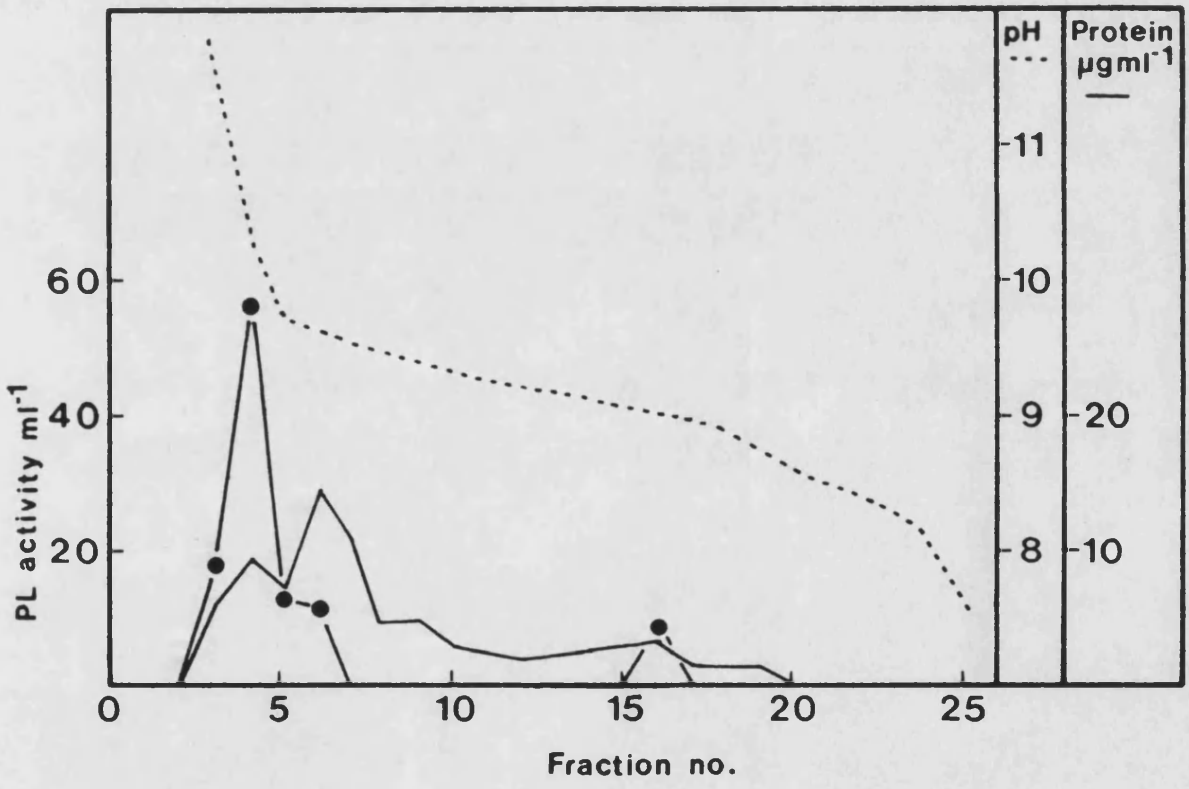


Plate 16

Polygalacturonase isozyme profiles of *V. albo-atrum* +Type and mutants.

(1) +Type; (2) C23; (3) 24d; (4); 34i; (5) 111b.

Fractions pH 8.5-11.0 of +Type and 24d PL from broad range IEF were resolved in a narrower pH gradient (8-10.5) to distinguish between the two major alkaline enzymes more clearly (Fig 59). The significant loss of the PL isozyme (pI 9.0) from 24d was thus confirmed (compare to Fig 49 in Results and Discussion 2.2.).

PG profiles of VAA +Type and mutants were obtained by flat-bed IEF in combination with NAPP activity staining (Materials and Methods 9.2.2). PG profiles from pectin cultures of +Type, 111b and 24d were equivalent in intensity and band number, reflecting the overall similarity in PG activity produced by these strains (Plate 16). C23 produced all of the isozymes, but the staining of this gel lane was generally weaker which presumably indicated an overall reduction in the release of the isozymes.

34i produced a single PG isozyme of identical pI to the enzyme produced basally by the +Type on glucose or CMC (Results and Discussion 2.2.1.).

3.2.5. Spore production, morphology and growth *in vitro*.

Genetical disorders in biochemical pathways involved with basic respiration would be shown phenotypically as reduced growth. Altered morphology could result from lesions affecting branching or cell wall synthesis. Colony growth and morphology was therefore assessed on glucose or sucrose/salts medium. Morphological changes would also reveal any pleiotropic effects resulting from pectinase mutations. Reduced conidial production would be of particular importance in vascular parasitism by VAA as this could lead to a decrease in the rate of colonisation *in vivo* and so this was also examined in culture.

Radial growth rates of +Type, isolate 111b and mutants C23, 24d, 34i were almost identical during 12 d on Czapek-Dox agar (Fig 60). Sporulation and hyphal morphology was also indistinguishable from the

MYCELIAL GROWTH AND CONIDIAL PRODUCTION BY V. ALBO-ATRUM +TYPE AND MUTANTS IN LIQUID AND SOLID MEDIA.

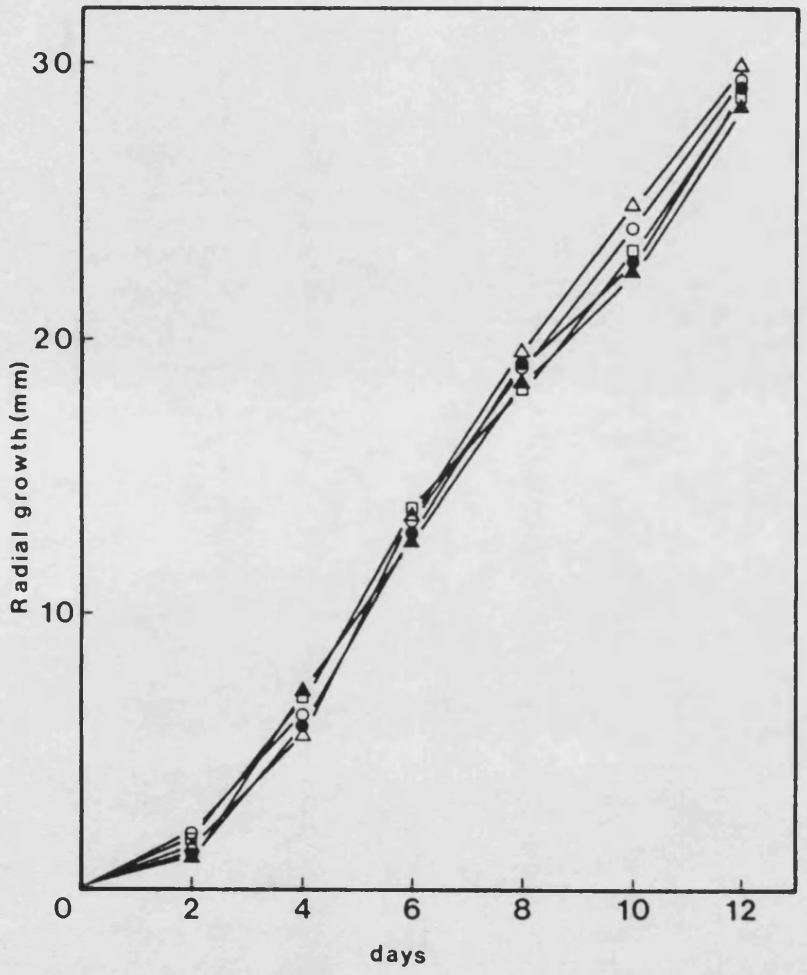


Fig 60. Radial growth on Czapek-Dox agar at 23C.
Mean results of 6 plates.

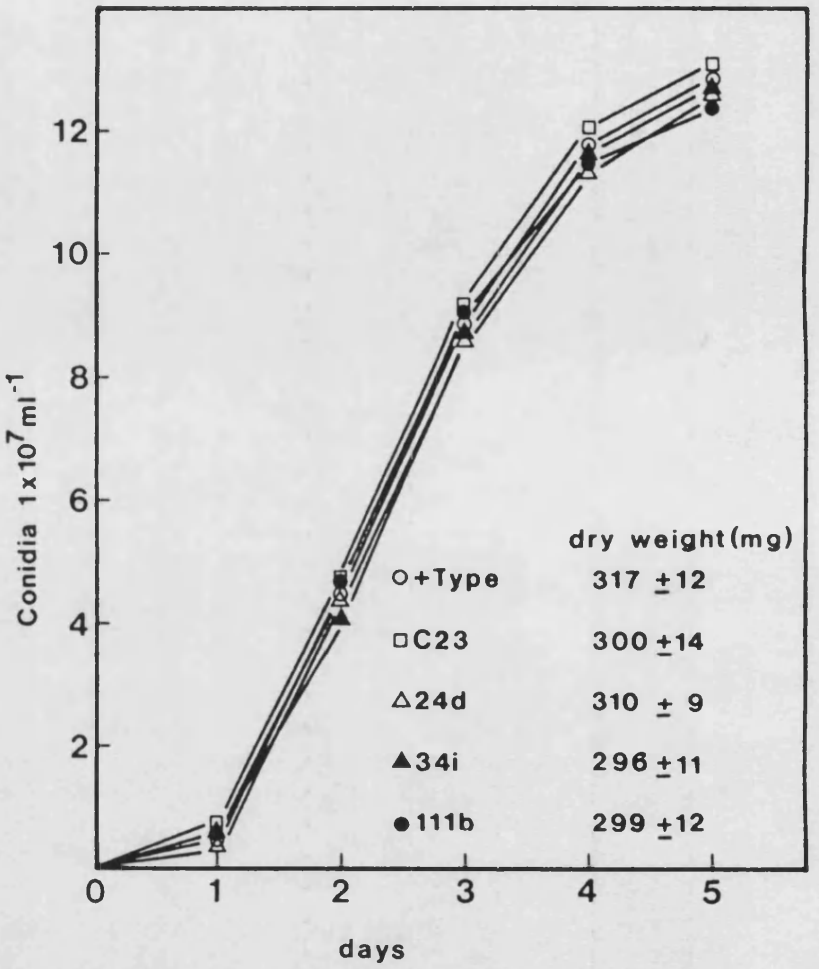


Fig 61. Conidial production and final mycelial dry weights in 5 d minimal salts cultures (100 ml) containing glucose (0.5 %, pH 6.5).
Mean results of 4 cultures.

+Type; all isolates were largely hyaline on Czapek-Dox although melanisation and thickening occurred in some hyphae after *c* 14 d.

Growth on PL selection medium (P8) was similar for all isolates apart from 34i which grew poorly, attaining a colony diameter of only 3.5 mm after 7 d, in contrast to 14 mm by the other mutant and +Type strains (Table 25). Similarly, 34i grew poorly on N5 selection medium, after 7 d colonies attained a diameter of 4 mm whereas those of the other strains were 11 mm across 7 d (Table 25).

Growth rate, hyphal morphology and conidial production of all five strains were very similar on glucose/salts medium over 5 d (Fig 61). Microscopic examination showed conidia of all strains were of similar morphology and size, ranging from 4.0-11.0 μm x 2-4 μm for each strain. Hyphal branching was no more pronounced in any of the isolates in liquid or solid medium. Furthermore, hyphal diameters which ranged from 2-3 μm in both medium were also indistinguishable between strains. It was concluded that because the isolates all grew equally well on simple carbon sources there were no basic changes to the metabolism of the mutants either from direct or pleiotropic effects of mutagenesis.

3.3. Pathogenicity and virulence of pectinase-deficient mutants.

The virulence and pathogenicity of the VAA mutants and 111b were compared to that of the +Type in an effort to correlate changes in pectinase production with ability to cause symptoms and colonise the host. In preliminary experiments, differences in epinasty, chlorosis and wilting were observed in plants infected with the +Type and the pectinase-mutants. In later experiments (reported below) attempts were made to quantify the data as far as possible, in order that statistical analyses could be undertaken. The raw data for symptom formation in experiments 1 and 2 are presented in Appendices 13 and 14; the statistical analyses are shown below in Tables 31 and 32.

Disease was assessed quantitatively in plants inoculated with the +Type and with the pectinase mutants, by scoring the number of leaves that were affected with each symptom as it developed. The results were analysed by a two-way analysis of variance with replication, between symptoms appearing on plants inoculated with the +Type on those infected with the mutants. 111b was also used in pathogenicity tests as a further positive control; various evidence from *in vitro* suggests that it can be considered as +Type. The extent of colonisation within plants was assessed by determining the % of infected vessels in hand sections of vascular bundles removed from mid-internodal regions 1, 3, 5 and 7 (Fig 62 and 63).

Epinasty and chlorosis developed on all the plants inoculated with VAA +Type and mutants but the severity and timing of these symptoms varied. Plants inoculated with the mutants C23, 24d and 34i were significantly less epinastic and chlorotic during the first 2-3 weeks of incubation (Tables 31 and 32; Plates 18, 19 and 20). Wilting induced by 24d and 34i was delayed by 3 d in experiment 2 (Table 32). Furthermore, the secretory mutant, C23, failed to induce wilting and caused only very slight symptoms.

Table 62. Two way analysis of variance between symptoms apparent on the lower 8 leaves of 6-8 week old tomato plants (cultivar GCR 26) inoculated with VAA +Type and mutants in April 1986. (Data normally distributed). ($p < 0.001$, ***; $p < 0.01$, **; $p < 0.05$, *; not significant, ns). See Appendix 13 for the raw data for experiment 1.

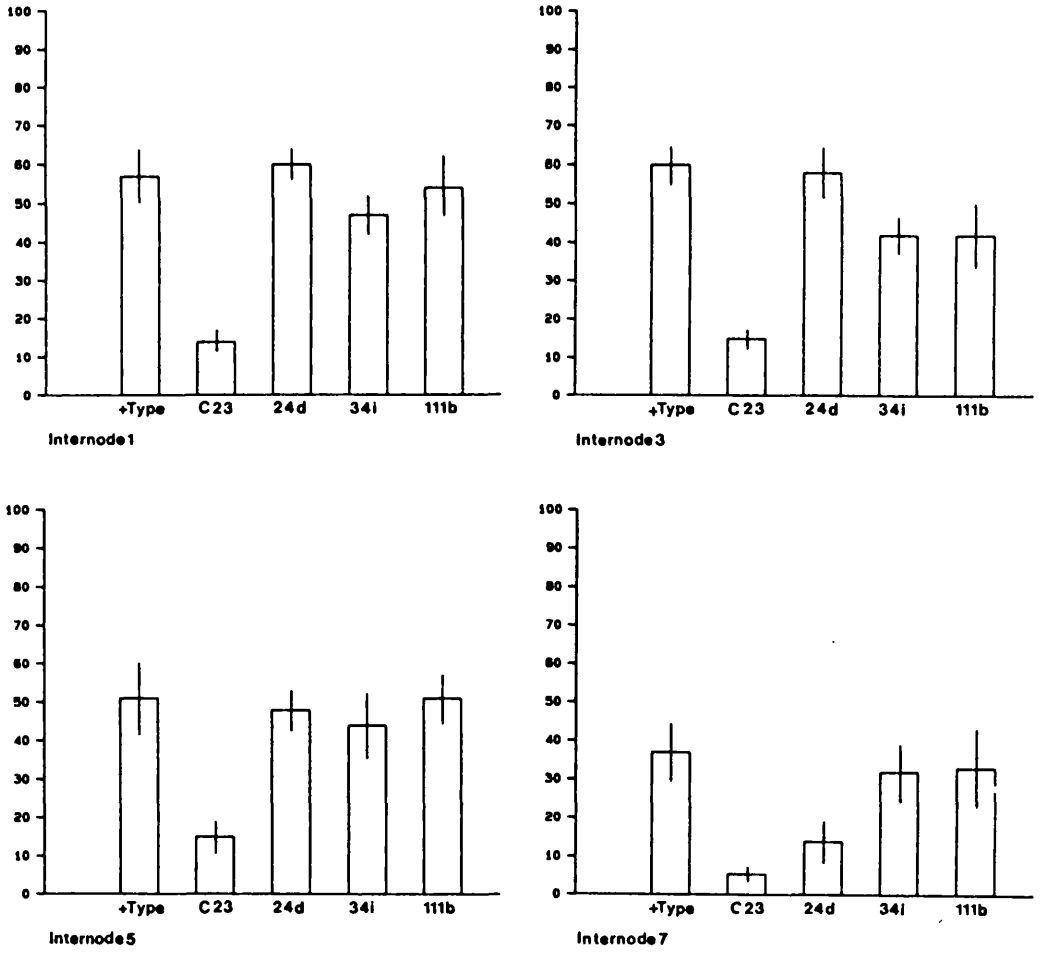
Epinasty		Days after inoculation				*a*
	Strain	13	15	17	20	23
	C23	*	*	*	*	*
	24d	**	***	ns	ns	ns
	34i	*	**	ns	ns	ns
	111b	ns	ns	ns	ns	ns
	Control *b*	***	***	***	***	***

Chlorosis		Days after inoculation				*a*
	Strain	15	17	20	23	25
	C23	ns	*	*	*	**
	24d	ns	*	*	*	ns
	34i	ns	*	*	*	ns
	111b	ns	ns	ns	ns	ns
	Control *b*	ns	***	***	***	***

Wilting		Days after inoculation				*a*
	Strain	17	20	23	25	26
	C23	ns	ns	***	***	**
	24d	ns	ns	*	ns	ns
	34i	ns	ns	*	ns	ns
	111b	ns	ns	ns	ns	ns
	Control *b*	ns	ns	***	***	***

a Seven plants were inoculated with 50 ml conidia (1×10^6 ml^{-1}) from 3d glucose salts cultures poured onto the soil surface around the base of each plant.
b Uninoculated.

Fig 62. PERCENTAGE OF VESSELS COLONISED BY V. ALBO-ATRUM +TYPE AND MUTANTS 23 DAYS AFTER INOCULATION. EXPERIMENT 1. See Appendix 13 for raw data concerning symptom development; statistical analysis in Table 31.



Colonisation assessed as the mean % of vessels containing hyphae in sections removed from the middle of internodes 1, 3, 5 and 7 from 5 plants.

Plates 17-21. Symptoms on tomato plants (cultivar GCR 26), 23 d after inoculation with *V. albo-atrum* +Type and pectinase-deficient mutants.

Experiment 1.

Quantitative assessment of symptoms and statistical analysis in Appendix 13 and Table 31. Quantitative assessment in Fig 62.

Plate 17. +Type and uninoculated control.

Plate 18. +Type and secretory mutant C23.

Plate 19. +Type and pectin lyase-deficient mutant 24d.

Plate 20. +Type and polygalacturonase/pectin lyase metabolic mutant 34i.

Plate 21. +Type and 111b (also +Type).

Plate 17

Plate 18

Plate 19

Plate 20

Plate 21

Each strain was reisolated from surface-sterilised segments of stem after incubation on Czapek-Dox agar as fluffy white mycelium which grew out of the cut ends of the vessels. The mutants had not reverted to the +Type as was evident from their reduced capacity to produce PL on P8 detection media plates.

From experiment 1 (Fig 62) it is apparent that all of the strains colonised the host plants to at least internode 7. The +Type, 111b and the mutants, 24d and 34i had colonised *c* 55 % of the vessels in internodes 1, 3 and 5. In contrast C23 was found in only *c* 10 % of vessels. Internode 7 was very sparsely colonised by C23 and only 5 % of the vessels were colonised. 24d showed reduced colonisation in internode 7 (12 %) whereas 34i and the +Type were still found in almost 40 % of vessels.

Although the levels of colonisation achieved by 24d and 34i were slightly less than the +Type, the standard errors overlapped and there was therefore no significant difference in colonisation. C23, however, colonised the plants to a significantly reduced extent. Bright sunshine prevailed during the second experiment and this increased the transpiration rate causing the plants greater water stress than in the first experiment, in which wilting did not occur until 23d and only when sunshine levels increased transpiration. Colonisation in plants incubated for 27-30 d (Fig 62) was more extensive than in plants incubated for 19-21 d (Fig 63). The ambient temperature in the greenhouse remained close to 20°C in both experiments.

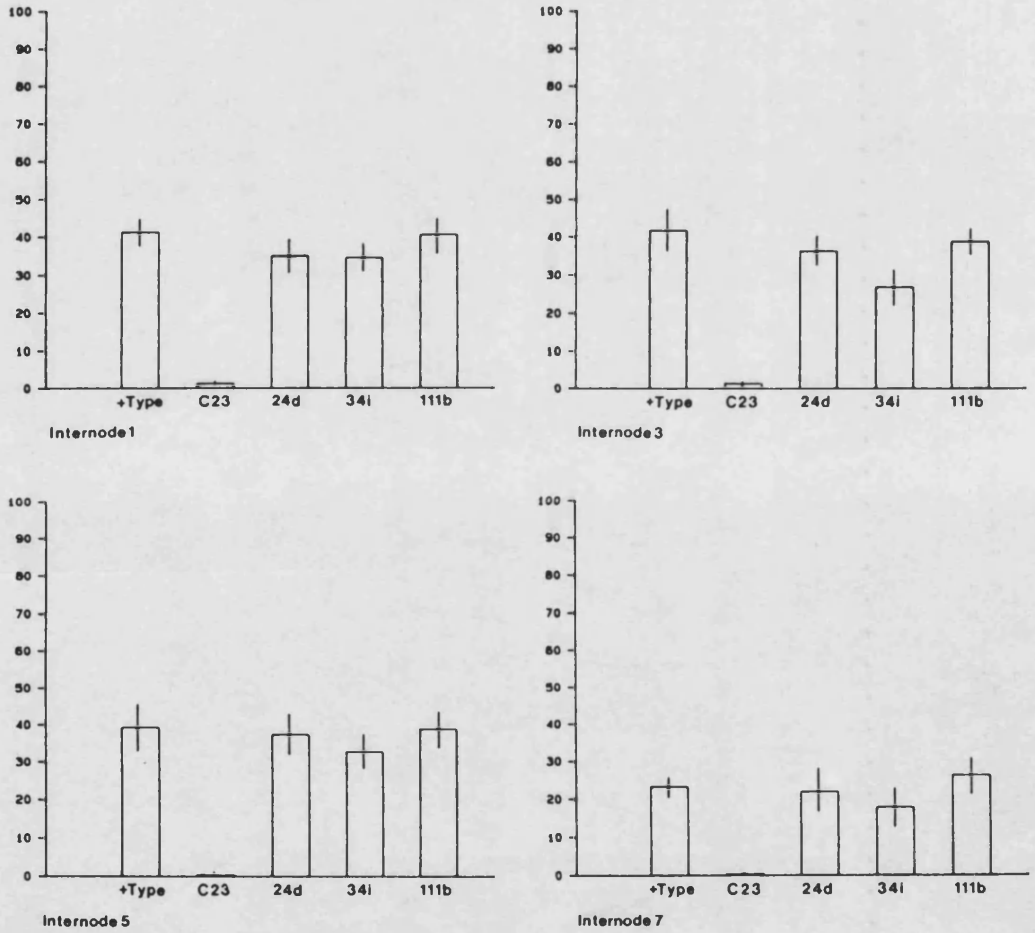
As in experiment 1, the plants infected with the mutants in experiment 2 were less chlorotic and epinastic, their overall appearance was healthier (Plates 23, 24 and 25), compared with +Type and 111b (Plates 22 and 26).

Table 63. Two way analysis of variance between symptoms apparent on the lower 8 leaves of 6-8 week old tomato plants (cultivar GCR 26) inoculated with VAA +Type and mutants in June 1986. (Data normally distributed). ($p < 0.001$, ***; $p < 0.01$, **; $p < 0.05$, *; not significant, ns). See Appendix 14 for raw data for experiment 2.

Epinasty		Days after inoculation *a*						
	Strain	11	13	14	15	16	17	18
	C23	ns	**	**	*	*	*	*
	24d	ns	*	*	ns	ns	ns	ns
	34i	ns	*	*	*	ns	ns	ns
	111b	ns	ns	ns	ns	ns	ns	ns
	Control *b*	ns	**	**	***	**	**	**
Chlorosis		Days after inoculation *a*						
	Strain	11	13	14	15	16	17	18
	C23	ns	*	*	*	*	*	*
	24d	ns	*	*	*	ns	ns	ns
	34i	ns	*	*	*	ns	ns	ns
	111b	ns	ns	ns	ns	ns	ns	ns
	Control *b*	ns	*	*	*	*	*	*
		Days after inoculation *a*						
Wilting	Strain	11	13	14	15	16	17	18
	C23	ns	*	**	**	*	*	*
	24d	ns	*	*	*	ns	ns	ns
	34i	ns	*	*	*	ns	ns	ns
	111b	ns	ns	ns	ns	ns	ns	ns
	Control *b*	ns	*	**	***	***	***	**

a Ten replicate plants were inoculated with 50 ml conidia (1×10^6 ml^{-1}) from 3 d glucose salts cultures poured onto the soil surface around the base of each plant.
b Uninoculated.

Fig 63. PERCENTAGE OF VESSELS COLONISED BY V. ALBO-ATRUM +TYPE AND MUTANTS 19 DAYS AFTER INOCULATION. EXPERIMENT 2. See Appendix 14 for raw data concerning symptom development; statistical analysis in Table 32.



Colonisation assessed as the mean % of vessels containing hyphae in sections removed from the middle of internodes 1, 3, 5 and 7 from 5 plants.

Plates 22-26. Symptoms on tomato plants (cultivar GCR 26), 18 d after inoculation with *V. albo-atrum* +Type and pectinase-deficient mutants.

Experiment 2.

Quantitative assessment of symptoms and statistical analysis in Appendix 14 and Table 32. Quantitative assessment in Fig 63.

Plate 22. +Type and uninoculated control.

Plate 23. +Type and secretory mutant C23.

Plate 24. +Type and pectin lyase-deficient mutant 24d.

Plate 25. +Type and polygalacturonase/pectin lyase metabolic mutant 34i.

Plate 26. +Type and 111b (also +Type).

Plate 22

Plate 23

Plate 24

Plate 25

Plate 26

3.4. Discussion of the isolation, characterisation and comparative virulence of the *V. albo-atrum* pectinase mutants and +Type.

Relatively few mutants were isolated from the large number of colonies screened, which suggests that either the selection system is failing to highlight their presence, that their occurrence is very rare, or because of the pectinase multiplicity, pectinase deficiency is phenotypically masked by the presence of other isozymes, unaffected by the mutation.

The selection media employed by earlier workers (eg, Puhalla & Howell, 1975) were shown to be inadequate (Materials and Methods 13). The media were unbuffered and as the pH tended to drift during incubation neither was exclusively detecting PG or PL.

The N5 and P8 selection media were however clearly mutually exclusive for PG and PL activity (Table 18), and therefore the probability of the absence of one pectinase species being masked by the activity of the other was almost entirely eliminated. N5 and P8 could provide the basis for a revised 'cup plate' media (Dingle, Reid & Solomons, 1953) for the semi-quantitative estimation of PG and PL activity. The two original media for detecting PG or PL would fail to maintain their respective optimal pH's of 5 and 8 because they were not buffered; also pH alters on autoclaving. In addition N5 and P8 media may be useful in comparing PG and PL production by a range of pathogens.

A specific medium for the selection of catabolite resistant mutants was not succesfully devised. However, N5 and P8 media may be useful in respect of CR mutants in addition to detecting pectinase-deficient mutants. As N5 and P8 media become rich in GALA and UGALA degradation products during incubation, at levels which could cause CR, it is likely that a CR^- mutant would be detectable because accumulation of high levels of pectinase must eventually occur in conditions of self-

catabolite repression (Collmer *et al.*, 1982b). The efficacy of the existing N5 and P8 media (both with and without a glucose supplement) for selecting CR^- mutants could only be fully evaluated by comparing clearing zones and growth of +Type with that of established CR^- mutants.

Understanding the mechanisms of PG and PL regulation in selection media was complicated by the failure of glucose to cause CR, whereas at similar levels in liquid culture complete repression occurs (Results and Discussion 1.1.1.). The behaviour of VAA on N5 and P8 media in the presence and absence of glucose may be explained as follows.

Initial production of PG on N5 media is clearly by induced synthesis as levels of PG were 100 fold greater on NAPP as sole carbon source than on (sucrose in Czapek-Dox; Table 18). Similarly, initial PL production on P8 media was also by induced synthesis. Further induction of both PG and PL on their respective media was probably restrained because of the high concentrations of GALA and UGALA that accumulated adjacent to the colonies. This situation is paralleled by that found in unrestricted liquid cultures containing pectin, where initial pectinase production, even at very low levels, is accompanied by an accumulation of oligo-uronides reulting from degradation of the polymer. Pectinase production does not assume high levels until this pool of accessible substrate has been utilised and CR is relieved. This may explain why the clearing zones produced by VAA on N5 were smaller than on N5 with glucose, as in both cases PG production was repressed, but the colonies comprised a greater fungal mass which was producing the enzyme basally. Differences in PL production on P8 and P8 with glucose were clearer. Basal levels of PL produced on glucose and CMC in liquid culture are undetectable (Results and Discussion 1.1.1.) and it followed that PL was detected at only very low levels on P8 containing glucose, which repressed

production. Relatively low levels of PL were also found in liquid culture under comparable conditions (Fig 9). Unfortunately attempts to identify the basal PG isozyme by flat bed IEF failed as the extract interfered with the focusing, even after purification by ammonium sulphate precipitation.

PL activity was further reduced around the colonies in P8 media because this enzyme is vulnerable to end-product inhibition, as evident from the increase in activity after dialysis of extracts (Table 23). The uronides in the colony extracts on N5 and P8 media (Plate 15) corresponded to the final degradation products detected in reaction mixtures of VAA PG with NAPP and PL with pectin (Cooper *et al.*, 1978).

It may be possible to prevent the effects of CR in solid selection media by including an insoluble inducer of enzyme synthesis. Finely ground host-cell walls could be suspended in an agar salts medium, buffered to optimise the activity of PG or PL (or indeed another CWDE). Activity could be detected around fungal colonies by pouring on an overlay of the relevant substrate, in a similar way to PG activity staining of flat bed IEF PAG plates (Materials and Methods 9.2.2.1.). Xylanase-deficient mutants of *Penicillium funiculosum* have been isolated in this way on ball-milled straw incorporated in an agar/salts medium (R. Hoffman pers. comm.). Constitutive mutants and constitutive non-repressible mutants could be obtained by adapting the agar medium and employing an overlay (Cooper, 1986a; Montenecourt *et al.*, 1979; Literature Review 2.4.).

The major reason for the rarity of PG^- and PL^- mutants is probably due to the large number of isozymes of each enzyme that are produced. As each enzyme is the product of at least one separate structural gene, the probability of simultaneously neutralising all of these is remote. If groups of the enzymes are jointly controlled by regulatory genes,

there would presumably be an increased chance of simultaneously removing some or all pectinase activity. Similar problems have been encountered by other workers with Ech *pel* genes which are being circumvented by the lengthy specific procedure of marker exchange mutagenesis (eg, *Collmer et al.*, 1985). The only specific PL^- mutant of VAA 24d, showed a reduced ability to produce 5 of the 6 PL isozymes, as identified by broad range IEF. The major alkaline PL isozyme (pI 10.6) was still produced at relatively high levels, which suggests that it is under separate control from the remaining 5 isozymes; presumably the 5 isozymes are under the overall control from a single regulatory gene and may be found to be clustered as are the *pel* genes of Ech (Kotoujansky *et al.*, 1985; Van Gijsegem, 1985). Conversely each isozyme may be under separate control. The *pel* genes a and b of Ech are each transcribed separately, in contrast to the premise that their known close association was due to co-transcription of the genes. These two genes have separate codons and their respective isozymes PGLa and PGLb produced independently by different *E. coli* subclones (Schoedel & Collmer, 1986).

The probability of inducing a mutation in a specific gene in VAA, eg for auxotrophy, is *c* 0.0001 % (Hastie & Heale, 1984). Therefore, if PG production was controlled by a single gene two of the 20 000 survivors screened on N5 media would be expected to be PG^-. However, no PG^- mutants were isolated and therefore production of the VAA PG isozymes is probably controlled by at least two separate regulatory genes. The large number of PG isozymes produced by VAA makes this organism unsuitable for the isolation of specific PG^- mutants, as attempts here have shown. From the results of Puhalla & Howell (1975) and Howell (1976), *V. dahliae* PG^- and PL^- mutants appeared at the rate of *c* 0.0001 % which is similar to the rate of attainment for auxotrophs (Hastie & Heale,

1984). The *V. dahliae* mutants were not fully characterised *in vitro*, but if as the results suggest, they were almost completely PG-deficient, *V. dahliae* would appear a more suitable pathogen for this kind of work. The reason for the successes with this pathogen may reflect the production of far fewer PG isozymes than by VAA (Results and Discussion 2.1.3.1.1, 2.2.1 and 2.4; Plate 6). From the PG profiles shown in Plates 7-10) it would appear that isolate 105 is the best candidate for mutagenesis, because it produces only one major PG isozyme.

It was expected that pectinase-deficient mutants might be outgrown or masked by +Type colonies on the selection media. In regard of this it was perhaps fortunate that the metabolic mutant 34i was isolated; this mutant may have been able to utilise non-uronide components of pectin, eg, arabinose or galactose. In future work it may be possible to isolate mutants similar to 34i by incubating survivors in a solution of GALA, containing an inhibitor (eg sodium azide), prior to inoculation of the selection media. By adopting this enrichment technique +Type spores capable of metabolising GALA would germinate and be killed, leaving behind the metabolic mutants which could be selected on a pectin selection medium containing a trace of glucose as a 'starter'. Mutants would grow slowly and produce small clearing zones similar to 34i on pectin media.

The specific loss of a potentially important isozyme may be masked on selection media by the presence of the remaining isozymes, and until the establishment of the use of marker-exchange mutagenesis with fungal pathogens, a more specific selection system is required. Antibodies raised against purified isozymes could be combined with fluorescent markers and applied to colonies grown on pectinase-inducing selection media; mutants lacking the isozyme would not fluoresce. Although a

feasible method, this would clearly be more complex than those adopted in this work. Furthermore such antibodies can in some cases, be used *in vivo* as markers of enzyme activity (eg O'Connell *et al.*, 1986).

New information concerning PG and PL regulation in VAA has been provided by the metabolic mutant 34i. It would appear that induction of PG and PL is under coordinate control because this mutant was simultaneously incapable of synthesising these enzymes in the presence of galacturonide inducers. GALA and UGALA residues released into the media by basal activity, (which were presumably mono and di-galacturonic acid, the final degradation products of these enzymes; Cooper *et al.*, 1978), were not utilised and furthermore failed to induce PG and PL. This indicates that GALA and UGALA are not the direct inducers of PG and PL synthesis as was suggested by Cooper & Wood (1975). This mutant is presumably incapable of converting galacturonides into an inducer; such an inducer could be produced during uronic acid metabolism which enables the +Type to grow on GALA and pectin as sole carbon source. $(GALA)_2$ and $(UGALA)_2$ metabolism in *E. chrysanthemi* involves the production of 4-deoxy-L-*threo*-5-hexosulose uronic acid (DTH) by an intracellular oligogalacturonide lyase. DTH is then isomerised, reduced and further metabolised to pyruvate which enters the citric acid cycle (Collmer *et al*, 1982b). As a similar mechanism probably exist in fungi (Rattigan & Ayres, 1977a and b) it is likely that 34i lacks one of the three key enzymes involved in the uronic acid pathway. If the intermediates of metabolism were available it should be possible to induce pectinase synthesis in 34i by circumventing the block in the pathway that produces the inducer and provides the fungus with energy for growth. It may be possible to determine whether OGL's are involved in the regulation of fungal pectinases as they are in mediating the production of PGL by soft-rot

bacteria (Collmer *et al.*, 1982; Collmer & Keen, 1986). An Ech OGL⁻ mutant, supplimented with an intermediate of (UGALA)$_2$ metabolism, DGH, produced +Type PGL (Collmer & Bateman, 1981). In the absence of the intermediate the mutant failed to produce PGL (Collmer *et al.*, 1982).

The above argument is dependent on uronide assimilation by VAA being a passive process. Before the assumed metabolic mutation can be fully substantiated the mode of uronide assimilation must be established. This can be achieved by determining the uptake of radio-labelled GALA by established mycelium both in the absence and presence of a metabolic inhibitor (eg sodium azide) or under different temperature regimes. If assimilation is a passive process similar levels of radioactivity would be expected in mycelial extracts from both treatments. In this way uptake of uronides by *S. fructigena* was shown to be largely passive (Rattigan & Ayres, 1977a & b), whereas assimilation by *R. secalis* is active and severely reduced by metabolic inhibitors (Ayres & Olutiola, 1973). Uptake of different metabolites may occur by independent mechanisms (Jennings, 1974) or occur coordinately (Rattigan & Ayres, 1977). If the latter is true of VAA, mutant 34i may be capable of uronide uptake, as it is capable of assimilating glucose, sucrose and metabolites of cell wall degradation.

It is unlikely that the failure of 34i to produce PG and PL in pectin culture was due to CR, imposed by the presence of high levels of GALA or UGALA, because the enzymes were also not produced on cell walls from which the release of free uronic acids remained minimal. Basal synthesis is independent of the presence of inducers for +Type and 34i, and involved the production of a single PG isozyme (Plate 6); in contrast, basal PL production apparently involved all six isozymes (Fig 58). The production of low levels of pectinases may have importance *in vivo* as they well release potential inducers from host cell walls to

induce synthesis of the major PG and PL isozymes (Cooper, 1977). The author is not aware of other examples of basal activity corresponding to a single isozyme.

Results obtained with mutant C23 have clearly shown that CWDE are selectively secreted, as this mutant has an impaired ability to release all of the extracellular enzymes tested. Secretory mutants are the most commonly isolated forms in studies with bacteria and fungi (Cooper, 1986a). Secretion of PGL by Ech is controlled by at least five Out genes, the products of which mediate the export of >90 % PGL (Andro *et al*, 1984). If secretion is mediated, by products of several genes in VAA which operate in series, one would expect to obtain many mutants that were phenotypically similar. If however the process was controlled by independent groups of genes, working in parallel, a range of different mutants would be expected to emerge. The secretion process has been shown to be highly complex, eg 23 complementation groups have now been identified in the secretory pathway of a yeast (Novick *et al.*, 1980). Some of these genes may be involved in post-translational modification of the enzymes and not directly in secretion *per se*. All of the extracellular enzymes tested were found to be secreted in lesser amounts by C23, thus it is apparent that a common secretory system was affected by the mutation rather than a mechanism exclusively involved in pectinase-secretion.

The three pectinase-mutants isolated here have also provided some important knowledge concerning the rôle and significance of PG and PL in vascular wilt disease. Pectic enzymes may have a rôle in penetration, colonisation and fungal nutrition within the host. As they can cause maceration and cell death *in vitro* (Cooper *et al.*, 1978; Cooper & Wood, 1980), these pectinase may cause host damage directly during pathogenesis. Alternatively, the indirect result of these enzyme

activities may lead to the release of toxic products from walls or numerous enzymes of potential physiological effects from the host walls which may be involved in pathogenicity or result in diverse side effects and symptoms (Cooper, 1984; Strand & Mussell, 1975; Strand *et al.*, 1976).

Estimates of vascular colonisation determined in experiments 1 and 2 (Figs 62 and 63), showed that all 5 isolates infected the host plants up to at least internode 7. Significantly, extensive colonisation was not achieved by the secretory mutant C23. This suggests that the overall deficit in extracellular CWDE reduced the ability of this isolate to invade and establish itself *in vivo*. Growth on cell walls and in other media was indistinguishable from that of +Type, thus the reduced pathogenicity of C23 is probably not based on insufficient nutrition derived from wall breakdown. The pathogen must encounter and penetrate numerous cell walls as it colonises the host (Cooper, 1983). CWDE are presumably important in breaking down these barriers and therefore, the limited ability of C23 to release extracellular CWDE should confer a disadvantage during colonisation.

Endo-pectinases are generally assumed to be of central importance in cell wall breakdown by facultative parasites. It is puzzling therefore, that 34i which lacks all but basal PG and PL was able to colonise the host to the same extent as the highly pectolytic +Type. This could be explained in several different ways. Firstly, basal levels of PG and PL may be sufficient to enable this isolate to penetrate and colonise host tissues. The pathogenicity of a mutant of Ech, which was deficient in OGL-1, was seemingly unaffected by its inabilities to produce +Type levels of PGL and utilise the degradation products (Collmer & Whalen, unpublished results, in Collmer *et al.*, 1982). It was concluded that induction of PGL *in vivo* is superfluous and pectate utilisation is

unnecessary. However, it is unlikely that PGL production is entirely redundant *in vivo*, and basal activity is itself enough to maintain virulence (Collmer *et al.*, 1982).

Secondly, it is not inconceivable that PG and PL were synthesised by 341 *in vivo* at induced levels because of the release of an inducer by host enzymes. Thirdly, the comparative avirulence of C23 suggests that pectinases are not the sole factors conferring ability to degrade host walls; other CWDE may have a critical rôle in wall penetration.

Wall degradation may involve the synergistic action of a combination of CWDE, exemplified by mutants of *S. fructigena* for which virulence in apple fruits was correlated with the joint involvement of α-L-arabinofuranosidase and PG (Howell, 1975).

It has been suggested that the extravascular phase of disease may be regarded as the 'determinative phase' of the disease (Talboys, 1958), based on the assumption that pathogen activity within the vascular system is constant. Interactions within the root extravascular system could limit the amount of mycelium entering the vascular system (Bishop & Cooper, 1983a). Although the endodermis was not the only point of entry into the vascular system in pea and tomato, it did appear to be especially effective at reducing the number of hyphae entering the stele of resistant cultivars, and may have the important effect of localising and preventing extensive establishment within the xylem. (Bishop & Cooper, 1983a). Furthermore this region may be important at limiting the progress of a weakly virulent isolate such as C23 because an insubstantial challenge to the host would allow time to curtail invasion through suberisation, lignification, or by the production of fungitoxic compounds. C23 was shown to produce comparatively low levels of C_4, C_8 and C_{14} esterases by API-ZYM assay. These or similar enzymes would be essential for breakdown of the suberised radial cell walls of

mature endodermal cells (Scott & Peterson, 1979) and may partly explain why C23 failed to colonise the host extensively.

Recent evidence from *E. coli* clones carrying plasmids coding for a number of pectinase genes of *E. carotovora* strongly suggests that an *exo*-PGL, 2 *endo*-PGL's and an *endo*-PG are regulated together and that the enzymes are interdependent for succesful pectolysis of potato tissue (Roberts *et al.*, 1986). If VAA PL isozymes also operate in concert, this may explain why 24d is less virulent than the +Type as the mutant produces only very low levels of 5 out of the 6 isozymes.

Ultrastructural studies of root invasion by hyphae of VAA have shown that enzymic wall degradation is very localised, involving minimal damage around penetration pegs and is therefore essentially biotrophic as host cells apparently remain undamaged (Bishop & Cooper, 1983a). This indicates that either the pathogen produces very low, perhaps basal, levels of pectinases during penetration or that the enzymes are largely hyphal bound. 34i would not be at any disadvantage during this stage of invasion as it produces both PG and PL at basal levels. Additionally, relative to growth, this isolate would appear to produce +Type levels of the esterases that are probably necessary for degradation of endodermal walls. Similarly, 24d would not be disadvantaged, if only basal levels of PL are involved at this stage of infection.

Vascular colonisation and breaching of the pit membranes at vessel ends in which penetration is apparently achieved by localised degradation of this pectin-rich middle lamella primary wall complex (Bishop & Cooper, 1983b; Pegg, Gull & Newsam, 1976). Presumably, basal pectinase or wall-bound activity is sufficient for penetration, because successful colonisation of the vascular system, was achieved by 34i.

Wall degradation, remote from direct hyphal-host contact, has been observed, and presumably results from extracellular CWDE activity and possibly pectinases. However, PG and PL have not been isolated from whole tomato plants but only from infected cuttings, (Bishop & Cooper, 1983a; Cooper & Wood, 1980; Mussell & Green, 1970). Gels which occlude xylem vessels often stain deeply with ruthenium red, which is indicative of exposed pectic material (eg Pierson, Gothoskar, Walker & Stahman, 1955). Clearly the mutants, 34i, C23 and 24d would be less able to cause direct damage in this way.

The xylem sap is low in nutrients and consists of inorganic salts and trace levels of amino acids and sugars (Pegg, 1981; Wood, 1961). It has been suggested that the action of pectinases may be of importance in providing the pathogen with metabolisable uronides, followng degradation of the vessel walls and pits (Howell, 1976). However, the metabolic mutant 34i provides evidence which contrasts strongly with this assumption. Firstly 34i succeeded in establishing itself in the host although it produced less than 10 % of +Type levels of extracellular PG or PL, (assuming that synthesis did not occur *in vivo* by an unknown host-mediated mechanism). Secondly, 34i is incapable of utilising the hydrolytic and lytic breakdown products from polygalacturonides in pit membranes. Nutrition during vascular colonisation may therefore be primarily through the utilisation of amino acids as indicated by Dixon & Pegg (1972); they showed that infection by VAA of some tomato varieties was linked to a reduction in the concentration of amino acids, particularly proline, in the sap. Amino-acid auxotrophic mutants could perhaps provide a useful clue to the relevance of amino acids for pathogen nutrition *in vivo*.

Secondary wall degradation may have provided nutrients for 34i, however ultrastructural studies have revealed limited breakdown of the

thickened xylem walls (Bishop & Cooper, 1983b). In addition, growth of 341 on cell walls was initially very slow perhaps until, exposure and degradation of non-uronide wall polymers, following removal of the surrounding pectic layer by the comparatively weak pectinase activity. It follows that dependence on secondary wall polymers would be expected to delay the rate of colonisation of xylem vessels, but this was not the case.

If extracellular pectinases are not primarily involved in either invasion or nutrition, then perhaps they play an important part in symptom formation. When tomato or cotton cuttings were exposed to purified *endo*-PG and PL from isolates of VAA, the leaves became chlorotic, necrotic and desiccated (Cooper & Wood, 1980; Mussell & Strouse, 1972), similar to the typical disease symptoms. The effects of the enzymes may have been indirect, such as in cotton wilt, described by Mussell (1973; see Literature Review 3.7.3.).

Some symptoms may be a side product of invasion but these may possibly benefit the pathogen as a result of overall weakening of its host and consequently of the expression of resistance. The host used in this study belongs to a cultivated tomato line. The host-parasite interaction is antagonistic and the host is intolerant of the parasite. Antagonism may not reflect the wild state, where under natural conditions the host is tolerant of the parasite and provides it with a niche until the end of the growing season. The contrasting parasitic cycle in the cultivated plant is rapid; the pathogen must continually invade new host plants if it is to survive. If plants are selected for field resistance, a balanced host-parasite interaction may be restored, so that a pathogen will survive in the host plants but will not cause severe symptoms. Moreover, changes in the pathogen population will not

disturb the equilibrium because there would be no selection pressure for an increase in virulence.

Symptom formation was delayed in cotton plants infected with non-pectolytic *V. dahliae* mutants (Howell, 1976) and similarly epinasty, chlorosis and wilting were delayed by several days in tomato plants inoculated with 34i and 24d. These results strongly suggest that pectinases and particularly *endo*-pectin lyase are involved in symptom induction. 34i produced lowest levels of PG and PL and caused less severe symptoms than the +Type; plants infected with the PL^- mutant 24d were markedly less chlorotic than those afflicted with the +Type.

More striking was the markedly reduced and/or delayed onset of leaf symptoms and wilting in plants colonised with the secretory mutant C23. The failure of C23 to cause wilting suggests the following. Firstly PG, PL and a range of CWDE may be involved in direct symptom formation or indirectly following the release of toxic substances from degraded host cell walls. Secondly this mutant is failing to release some unknown virulence factor. Thirdly symptom formation may be correlated to mycelial mass and colonisation.

These results highlight the benefit of using defined mutants to establish correlations between putative determinants of pathogenicity and/or virulence with infection and symptom formation.

General Discussion

The use of mutants for determining the significance of pectinases in pathogenesis has shown the potential of a genetical approach. However the work has also exposed the vulnerability of the conventional mutagenic and selection systems, that are essential for obtaining defined mutants.

Firstly, the choice of pathogen must be made with care. A model organism must represent a well studied host-parasite interaction in which CWDE have been implicated be amenable to solid and liquid culture, produce pectinases *in vitro* and have uninucleate spores. Furthermore the number of pectinase isozymes must be minimal. A suitable candidate that meets these requirements is *V. dahliae* isolate 105 which possesses only one major PG isozyme. For gene mapping purposes this fungus could be useful, as a transfer of markers between isolates has been achieved many times *via* the parasexual cycle (Clarkson & Heale, 1985b; Hastie & Heale, 1984). However it is necessary to produce appropriate auxotrophs to force the cross. This, in addition to inducing pectinase-deficiency, would make the work very laborious with the additional possible problem of effects of auxotrophy on pathogenicity. More useful would be a pathogen with a sexual stage, eg *Fusarium solani f. sp. pisi* which is currently under investigation here. The sexual stage of this pathogen has been used to determine the relevance of pisatin demethylation during pathogenesis (Yoder *et al.*, 1986). Furthermore, the possibility of isolating and cloning fungal pectinase genes is evident following manipulation of the pisatin demethylase gene of the sexual stage of this Ascomycete, *Nectria haematococca* (Cooper, 1986b; Yoder *et al.*, 1986). With the discovery of high frequency transforming vectors in *A. nidulans* (Ballance &

Turner, 1986), the cloning of fungal virulence genes will perhaps become more commonplace.

These latter approaches will hold the key to determining the importance of pectinases in a variety of fungal diseases of plants. Whereas a challenge from soft-rot bacteria might be considered almost equivalent to immersion of host tissue in pectinases, invasion by a hemibiotrophic fungus involves more subtlety and a prolonged delay in pectinase synthesis. The enigmatic strategy of hemibiotrophs makes them and the rôle of their pectinases of particular interest. Thus it would be worthwhile pursuing the infection strategy of the well studied *C. lindemuthianum* system, a sexual stage of which has been recently reported (Batista & Chaves, 1982).

Appendix 1. The formula for Medium X for the culture of *Botrytis* species, (Last & Hamley, 1956).

Part 1.

20 g Oxoid # 3 agar dissolved in 750 ml d. H_2O.

Part 2.

2 mg mycological peptone.

10 g glucose.

3 g casamino acids (casein hydrolsate).

6 g $NaNO_3$.

5 g KH_2PO_4.

0.5 g KCl.

0.5 yeast RNA.

250 ml d. H_2O.

Parts 1 and 2 were mixed after autoclaving (121°C, 15 min).

Appendix 2 . Growth of B. allii in different carbon sources; number and size of pellets produced in liquid culture.

Carbon source	Time (d) after inoculation [a] 1 pellets no.	1 pellets size (mm)	2 pellets no.	2 pellets size (mm)	4 pellets no.	4 pellets size (mm)	7 pellets no.	7 pellets size (mm)	10 pellets no.	10 pellets size (mm)	14 pellets no.	14 pellets size (mm)
Pectin, pH 5.0	-	< 1	1-200		200	5	200	5	200	5	200	5
Pectin. pH 8.0	-	< 1	1-200	2	200	5	200	5	200	5	200	5
Pectin/glucose, pH 5.0	-	< 1	1-200	3	2-300	6	2-300	6	2-300	6	2-300	6
Pectin/glucose, pH 8.0	-	< 1	1-200	3	2-300	6	2-300	6	2-300	6	2-300	6
Glucose, pH 5.0	-	< 1	1-200	3	2-300	< 1	2-300	2	2-300	2	2-300	3
Glucose, pH 8.0	-	< 1	1-200		2-300	2	2-300	3.5	2-300	3.5	2-300	4
CMC, pH 5.0	-	< 1	1-200	0.5	2-300	0.5	2-300	2	2-300	2	2-300	2
CMC, pH 8.0	-	< 1	1-200	1	2-300	1.5	2-300	1.5	2-300	3	2-300	3
Onion cell walls, pH 5.0	-	-	1-200	1	large aggregates formed, thus pellet size and frequency							
Onion cell walls, pH 8.0	-	-	1-200	1	could not be assessed							

a Conditions and experimental details in Results and Discussion 1.1.2. (Table 6 and Figs 14-23).

Appendix 3 . Production of polygalacturonase by B. fabae on different carbon sources.[a]

Carbon source and buffer pH	Time (h) 6.5 pH (final)	6.5 PG[b] (RVU)	48 pH (final)	48 PG (RVU)	96 pH (final)	96 PG (RVU)	final dry weight[c] (mg)
Glucose, pH 5.0	5.0	0	5.2	0	5.0	0	85.0
Glucose, pH 8.0	8.0	0	7.8	0	7.6	0	96.0
Glucose/asparagine, pH 5.0	5.5	0	6.6	0	8.2	0	150.0
Glucose/asparagine, pH 8.0	8.0	0	8.5	0	8.4	0	180.0
Pectin, pH 5.0	5.2	6.0	5.0	81.0	5.4	100.5	95.0
Pectin, pH 8.0	7.8	0	7.4	2.5	7.3	3.5	68.0
Pectin/asp., pH 5.0	5.5	0	5.6	0	7.0	0	80.0
Pectin/asp., pH 8.0	7.7	0	8.1	0	8.4	0	92.0
Pectin/glucose, pH 5.0	4.5	0	5.2	0	5.3	0	170.0
Pectin/glucose, pH 8.0	7.9	0	7.5	0	7.4	0	220.0

a All carbon sources provided at 1 % (w/v); buffered at pH 5.0 (MES, 0.05 M) or pH 8.0(HEPES, 0.05 M). 100 ml of media inoculated with 10^7 spores of B. fabae from 10 d YEP plates. Acidic media were adjusted to pH 5.0 prior to inoculation.
b PG assayed viscometrically; PL was not detected in any culture by TBA assay.
c Mycelial dry weights determined by collection on dried preweighed Whatman 1 filter paper and heating at 70 C to constant dry weight.

Appendix 4.1. Inner onion bulb epidermis infected with *B. allii* showing esterase activity on germinating spore and infection hypha 24 h after inoculation.

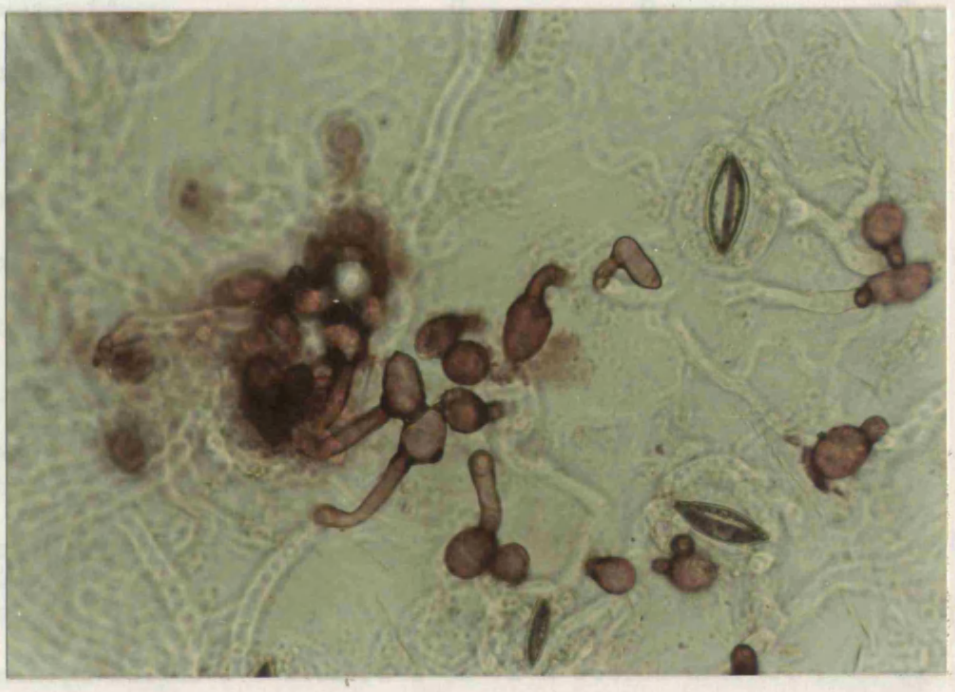
Inoculation conditions in Materials and Methods 16.2. Details of histochemical enzyme staining in Materials and Methods 17.4.

Appendix 4.2. Broad bean leaf epidermis infected with *B. fabae* showing esterase activity on germinating spores and infection hyphae 24 h after inoculation.

20 µl droplets containing 2 x 10^4 spores were placed on the adaxial surface of broad bean leaves, detached from 6 week old greenhouse grown plants. The leaves were incubated on moist filter paper in petri dishes at 18°C. Details of histochemical enzyme staining in Materials and Methods 17.4.

Appendix 4·1

Appendix 4·2



Appendix 5. Variety of enzymes produced by *B. allii* in liquid culture and in onion tissue as determined by API-ZYM strips.

Enzyme	pectin *a*	cell walls *b*	onion tissue *c*
Alkaline phosphatase	1	1	2
Esterase (C_4)	2	2	2
Esterase, lipase (C_8)	3	3	2
Lipase (C_{14})	1	1	1
Leucine arylamidase	2	1	2
Valine arylamidase	1	1	1
Cysteine arylamidase	1	1	1
Trypsin	1	1	1
Chymotrypsin	0	0	0
Acid phosphatase	3	3	2
Phosphoamidase	3	3	2
α-galactosidase	3	3	2
β-galactosidase	3	3	2
β-glucuronidase	0	0	0
α-glucosidase	3	1	1
β-glucosidase	3	3	1
N-acetyl-β-glucosaminidase	4	2	2
α-mannosidase	1	1	2
α-fucosidase	0	0	0

Representative results of three replicate strips. Activity in arbitrary units (0-5).

a Ammonium sulphate concentrate from 7 d pectin/salts culture (pH 5.0, MES 0.05 M).
b Ammonium sulphate concentrate from 7 d onion cell wall culture (pH 5.0, MES 0.05 M).
c Ammonium sulphate concentrate of 72 h lesion extract from onion mid bulb.

Cont...

Appendix 5. cont... Enzyme activities tested and substrates on API-ZYM strips. *a*

Enzyme	substrate
Alkaline phosphatase	2-naphthyl phosphate
Esterase (C_4)	2-naphthyl butyrate
Esterase, lipase (C_8)	2-naphthyl caprylate
Lipase (C_{14})	2-naphthyl myristate
Leucine arylamidase	L-leucine-2-naphthylamide
Valine arylamidase	L-valyl-2-naphthylamide
Cysteine arylamidase	L-cystyl-2-naphthylamide
Trypsin	N-benzoyl-DL-arginine-2-naphthylamide
Chymotrypsin	N-glutaryl-phenylamine-2-naphthylamide
Acid phosphatase	2-naphthylphosphate
Phosphoamidase	napthol-AS-B_1-α-D-phosphodiamide
α-galactosidase	6-Br-2-naphthyl-α-D-galactopyranoside
β-galactosidase	2-naphthyl-β-D-galactopyranoside
β-glucuronidase	napthol-AS-B_1-β-D-glucuronic acid
α-glucosidase	6-2-naphthyl-α-D-glucopyranoside
β-glucosidase	6-Br-2-naphthyl-β-D-glucopyranoside
N-acetyl-β-glucosaminidase	L-naphthyl-N-acetyl-β-D-glucosaminidine
α-mannosidase	6-Br-2-naphthyl-α-D-mannopyranoside
α-fucosidase	2-naphthyl-α-L-fucopyranoside

a See Materials and Methods 6.6. for experimental details.

Appendix 6. Variety of enzymes produced by *B. fabae* in liquid culture containing broad bean cell walls as determined by API-ZYM strips.

Enzyme	Activity (0-5) (*a*)
Alkaline phosphatase	1
Esterase (C_4)	1
Esterase, lipase (C_8)	2
Lipase (C_{14})	2
Leucine arylamidase	1
Valine arylamidase	1
Cysteine arylamidase	1
Trypsin	1
Chymotrypsin	0
Acid phosphatase	2
Phosphoamidase	1
α-galactosidase	1
β-galactosidase	1
β-glucuronidase	2
α-glucosidase	1
β-glucosidase	2
N-acetyl-β-glucosaminidase	1
α-mannosidase	0
α-fucosidase	0

Representative results of three replicate strips. Activity in arbitrary units (0-5).

a Samples removed from 7 d bean cell wall culture (0.5 % w/v, pH 6.5, unbuffered). Growth was rapid and all cell wall particles were dedraded or bound to the mycelium by 7 d. Final pH 5.8.

Appendix 7. Effect of inoculum concentration of *B. allii* conidia on the outcome of infection of onion epidermis.

conidia concentration ml^{-1}	5×10^3	5×10^4	5×10^5
number of conidia in 20 µl inoculum droplet (*a*)	100	1000	10 000
symptoms absent	39	14	0
limited lesions	6	14	9
spreading lesions (*b*)	4	20	43
Total inoculation sites	49	48	52
% spreading lesions	8	42	83

a Segments (2 x 4 cm) of mid-bulb onion scales were placed on moist filter paper in large petri dishes (14 cm diameter) and inoculated with conidial suspensions removed from 10 d old YEP plates of *B. allii*.

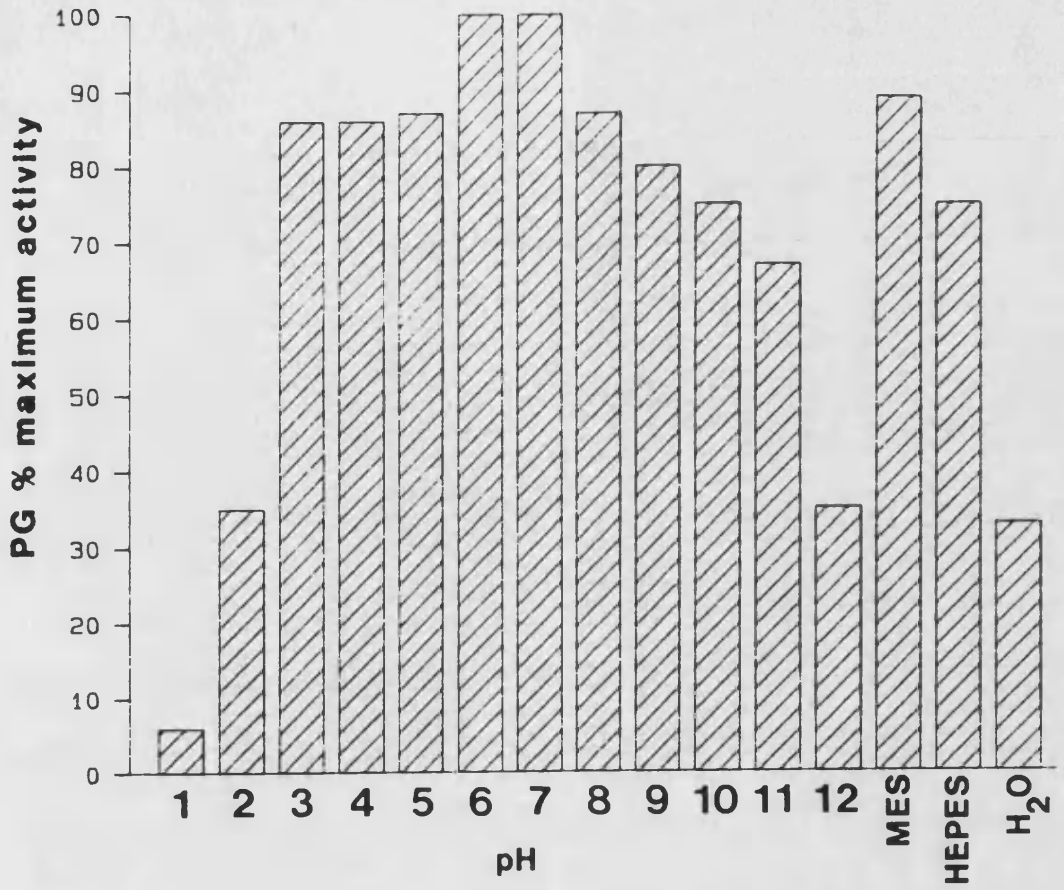
b After 5 d the infection had clearly spread beyond the site of inoculation.

Appendix 8. Effect of pH on the stability of *B.allii* PG and PL.

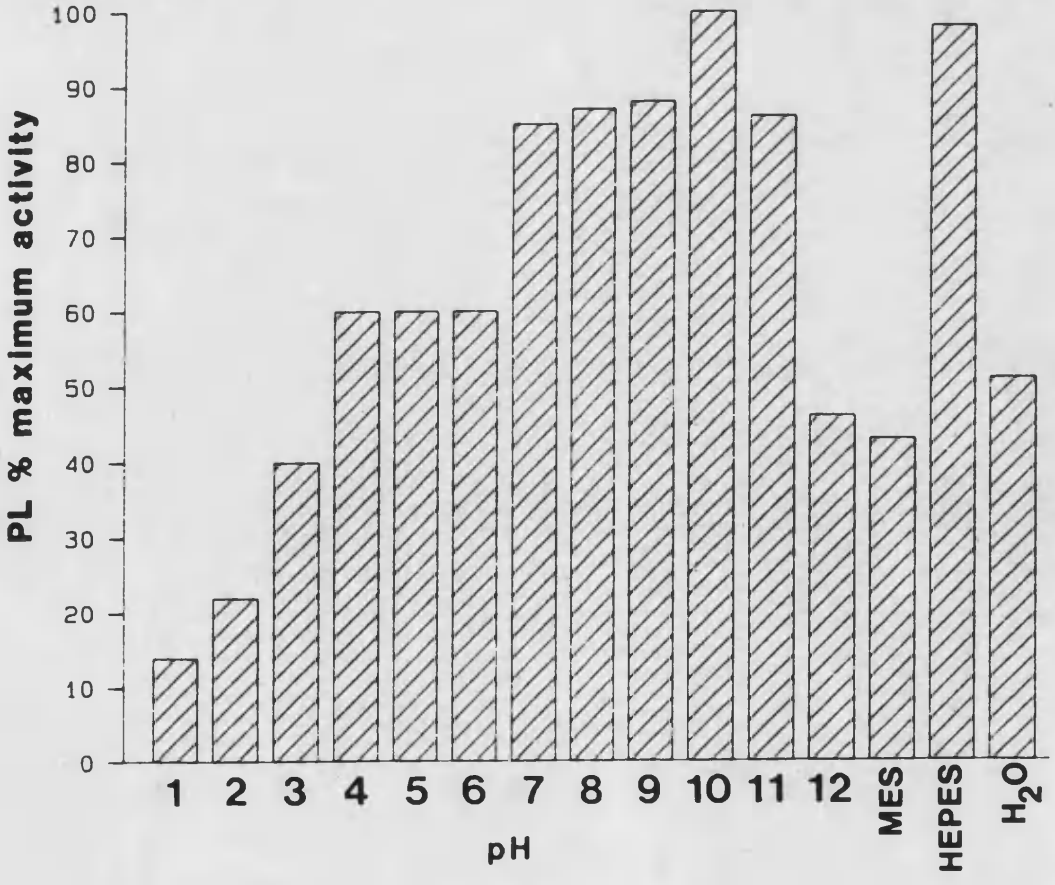
PG and PL may lose activity in certain adverse pH conditions. Excessive losses in activity may be avoided in liquid and solid media by creating optimal pH conditions. 2 ml of PG and PL were incubated with 2 ml of Universal buffer (pH 1-12), MES (pH 5.0, 0.1 M), HEPES (pH 8.0, 0.1 M) and d.H_2O (pH 4.7) for 24 h. Aliquots were dialysed overnight against 200 vol d.H_2O at 4°C and assayed for PG and PL activity.

PG was most stable over a wide range from pH 3-9 but highest activity was retained at pH 6-7 (Appendix 8.1.). In contrast PL was most stable in alkaline conditions between pH 7-11 (Appendix 8.2.), optimally at *c* pH 10. Buffer effects were minimal as both PG and PL were almost as stable, at equivalent pH, in the MES and HEPES as in Universal buffer. Both enzymes were less stable in d.H_2O.

Appendix 8·1



Appendix 8·2

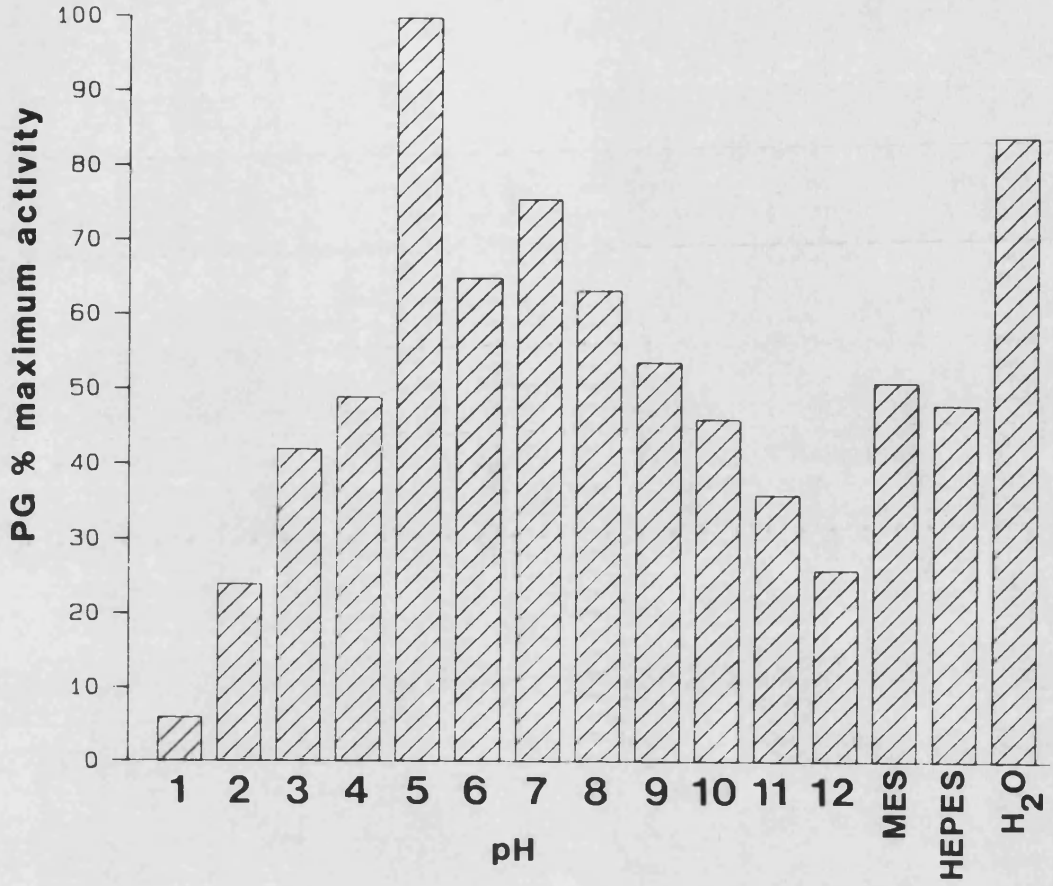


Appendix 9. Effect of pH on the stability of *V. albo-atrum* PG and PL.

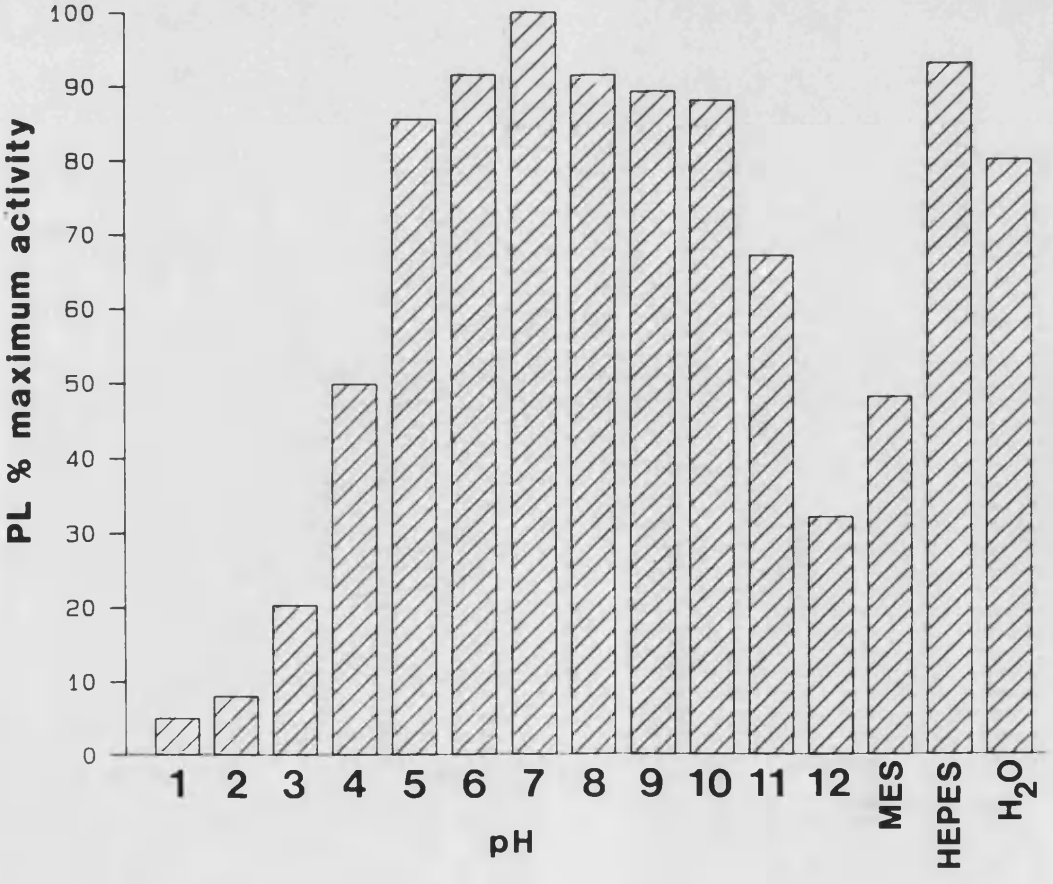
2 ml samples of PG and PL were incubated with Universal buffer, MES, HEPES and d.H_2O as described previously for *B. allii* PG and PL (Appendix 8.).

PG is most stable over a narrow range from pH 5-8 (Appendix 9.1.), but highest activity remained at pH 5. In contrast PL is most stable in a broad range from pH 5-11, optimally at pH 7-8 (Appendix 9.2.). PG and PL are generally more stable in Universal buffer than MES and HEPES, therefore activity losses are presumably attributable to buffer effects. Both enzymes remained very stable in d.H_2O (pH 4.7).

Appendix 9·1



Appendix 9·2



Appendix 10. Lesion development by five *B. allii* isolates on onion bulbs 5 and 10 d after inoculation. *a*

Isolate	5 d		10 d		
	Radial growth (mm) *b*	Sporulation	Radial growth (mm)	Sporulation	Depth of infection (no. of scales) *c*
2068	11.2	++	13.1	++	3
2070 *d*	10.2	+++	15.6	+++	4
3543	6.2	++	8.3	++	2
3669	8.4	++	11.0	+++	3
3670	12.1	++	16.1	+++	3

Mean results of 5 replicates.

a 5 x 5 mm cubes were cut and removed from 10 d old YEP plates. Single cubes from plates of each isolate were placed beneath 5 x 5 mm flaps cut into the outer bulb scales of onion bulbs. The bulbs were placed in sandwich boxes and incubated at 18°C. Lesions developed from the cut edges of these flaps. Sporulation occurred on the surfaces of the bulb scales and at the cut edges of the flaps.

b Radial growth measured from the flap edge.

c The depth of infection was determined by bisecting the bulbs after 10 d and counting the number of scales showing symptoms.

d The symptoms of bulb infection by isolate 2070 are illustrated in Plate 10.1.

Appendix 10·1

Onion bulbs infected with *B. allii* isolate 2070 10 d after inoculation. Inoculation and experimental conditions are described in Appendix 10.

Appendix 11. Nuclear frequency of *B. allii*, *B. fabae* and *B. squamosa* spores stained with DAPI.

No. nuclei	*B. allii*	*B. fabae*	*B. squamosa*
1	54	0	0
2	37	0	0
3	4	0	0
4	0	0	0
5	0	0	0
6	0	0	0
7	0	0	2
8	0	4	1
9	0	13	3
10	0	5	4
11	0	10	3
12	0	7	5
13	0	16	2
14	0	9	4
15	0	5	6
16	0	13	2
17	0	19	4
18	0	7	5
19	0	14	3
20	0	3	3
21	0	5	0
22	0	0	3
23	0	7	3
24	0	5	6
25	0	4	2
26	0	6	0
27	0	0	0
Total	95	152	61
Mean no. nuclei	1.47	16	16.7

Spores were removed from 10 d MX plates and suspended in DAPI (Hooley *et al.*, 1982). After 2 h incubation at 30°C the spores were viewed with a Leitz Orthoplan microscope under UV. Many of the *B. squamosa* spores were multicellular with up to 4 components.

B. fabae and *B. squamosa* produced multinucleate spores ranging from 9-27 nuclei spore^{-1} (Appendices 11.5-11.8). These two isolates are clearly unsuitable for mutagenic treatment as the multinucleate nature of the spores would favour complementation and heterokaryosis. *B. allii* was clearly more suitable as it produced mainly uninucleate spores although almost 40 % of them were binucleate Appendice s 11.1-11.4).

Cont...

Appendix 11. cont....

Menzinger (1965) reported *B. allii* to have an average of 1.33 nuclei spore^{-1} whereas Threlfall (1971) reported that *B. allii* produced at uninucleate conidia at a frequency of 80 % on YEP media.

Isolate 2070 was therefore grown on MX and YEP media to determine whether the high frequency of binuclear spores was either an effect of the media or a reflection of strain difference.

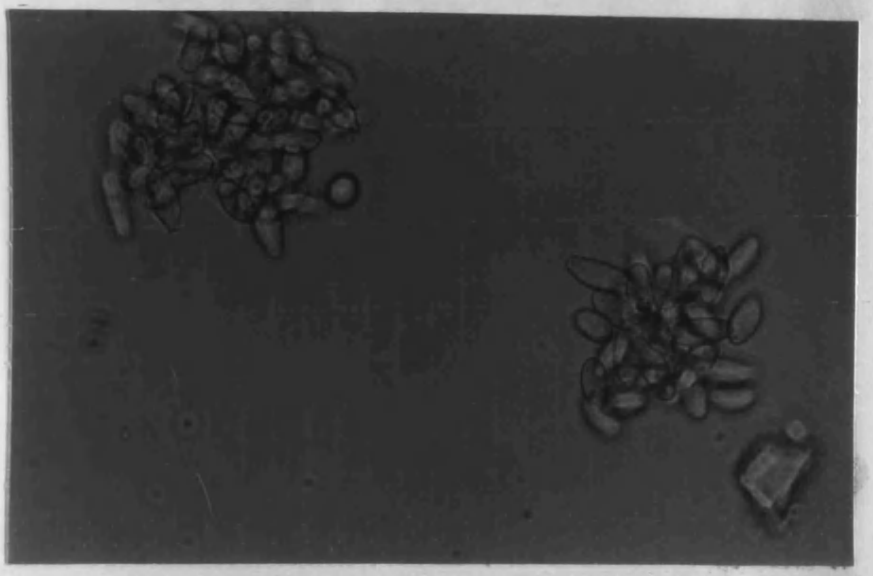
Appendix 11.1. Nuclear frequency of *B. allii* spores produced on MX and YEP media, as determined by DAPI staining.

Media	Nuclear frequency of spores			
	1	2	3	Total
MX	59	41	2	102
YEP	89	13	0	102

A χ^2 test of this data showed there to be a significant difference in the ratio of uninucleate:binucleate spores produced on the two media. Clearly MX media promoted the formation of multinucleate spores and YEP is a more suitable media for culturing spores for mutagenesis. The mean number of nuclei spore^{-1} was only 1.13 on YEP, in contrast to 1.47 on MX. When *B. fabae* was also grown on YEP, no significant difference was found in the range or the average number of nuclei spore^{-1}.

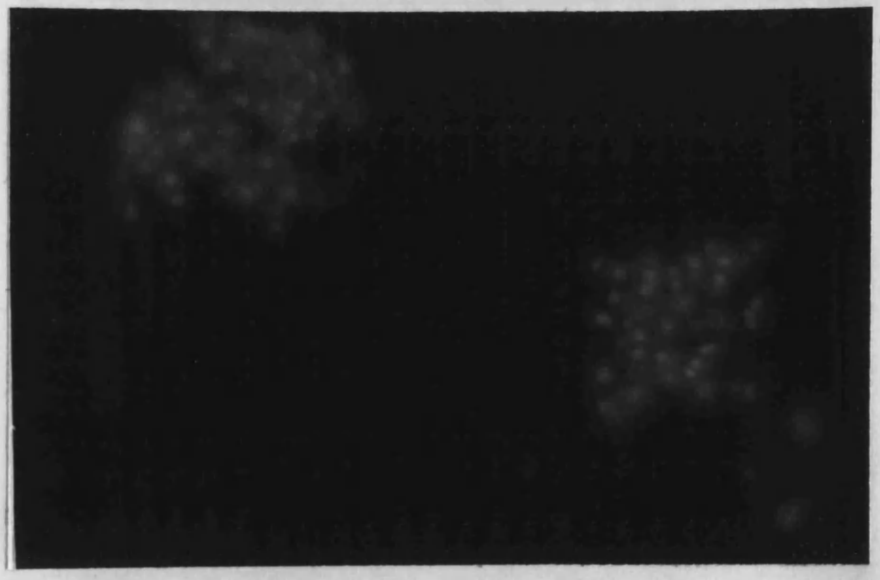
DAPI staining of *B. allii* spores.

Appendix 11.1



Light micrograph of a clump of DAPI stained *B. allii* spores.

Appendix 11.2



Fluorescence micrograph of DAPI stained spores shown in Appendix 11.1. The majority of the spores are uninucleate; however some are clearly binucleate.

DAPI staining of *B. allii* spores.

Appendix 11.3

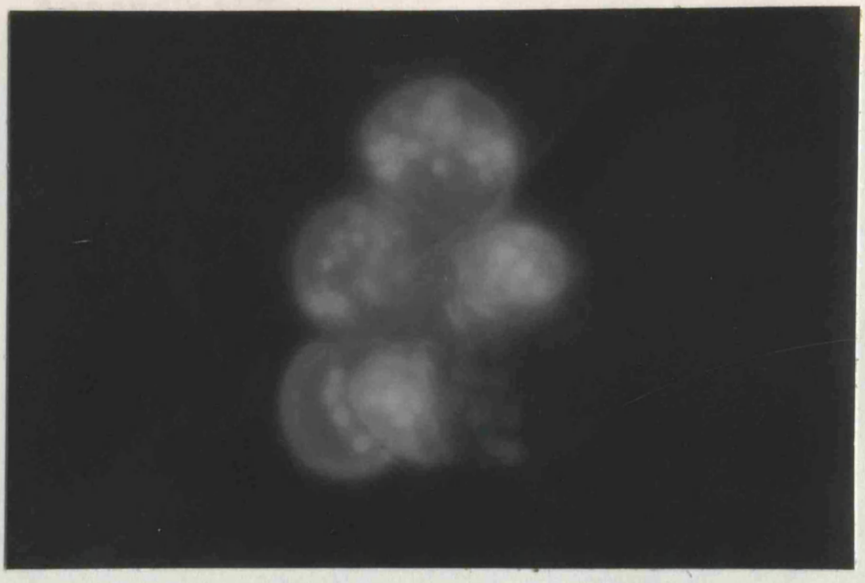
Light micrograph of a DAPI stained *B. allii* spore.

Appendix 11.4

Fluorescence micrograph of the spore shown in Appendix 11.3.

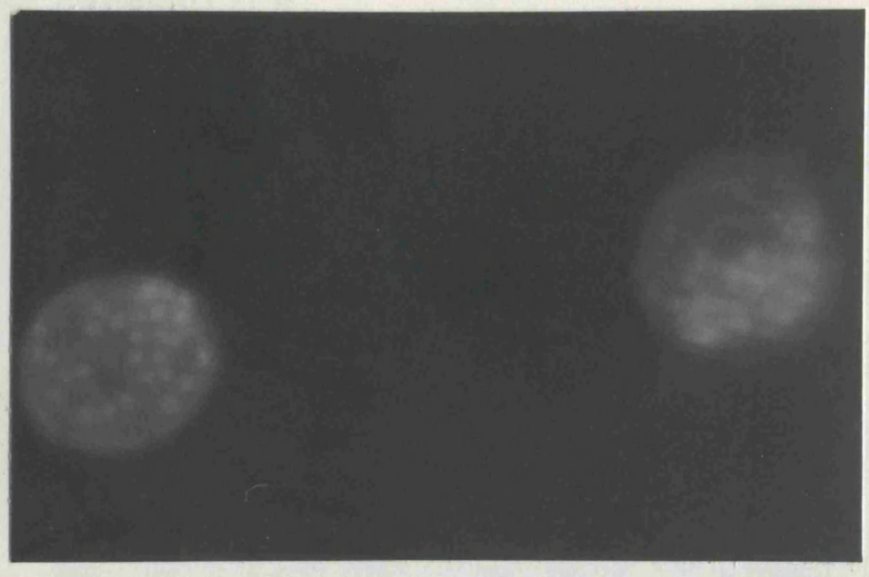
DAPI staining of *B. fabae* spores.

Appendix 11·5



Fluorescence micrograph of a clump of DAPI stained *B. fabae* spores.

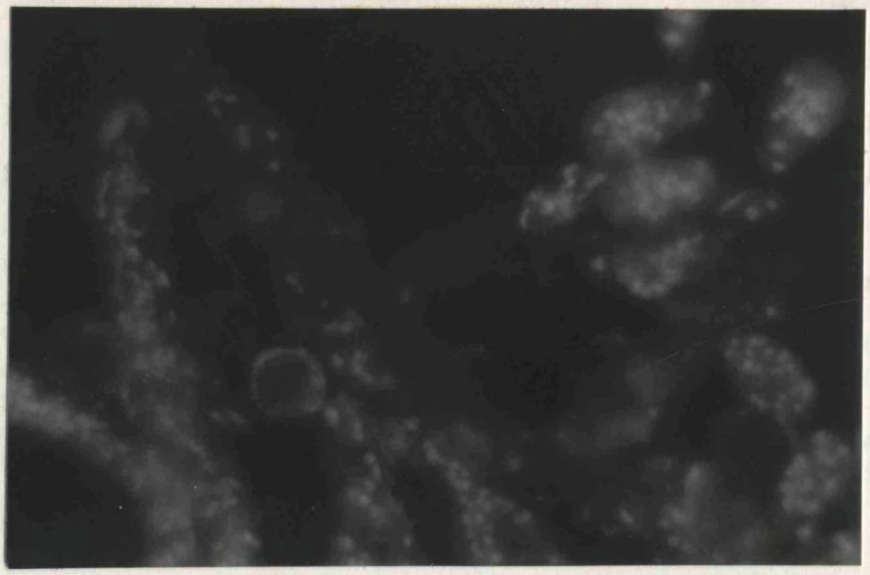
Appendix 11·6



Fluorescence micrograph of DAPI stained *B. fabae* spores.
Note at least 27 nuclei are visible in the left hand spore.

DAPI staining of *B. squamosa* spores.

Appendix 11·7



Fluorescence micrograph of a clump of DAPI stained *B. squamosa* spores, conidiophores and aerial hyphae.

Appendix 11·8

Fluorescence micrograph of a DAPI stained *B. squamosa* spore. This spore consists of 4 multinucleate cells.

Appendix 12. Citrate buffer; its usefulness in culture, its utilisation and inability to cause catabolite repression of polygalacturonase synthesis by *V. albo-atrum*.

Culture	Buffer molarity		pH	Dry weight (mg)	PG (RVU)
	Initial	Final			
Pectin	0	0	4.4	203	130.7
Pectin + citrate	0.005	0.004	4.5	235	212.5
Pectin + citrate	0.05	0.042	4.5	327	1850.4

1×10^7 spores of VAA from Czapek-Dox plates were added to four replicate 100 ml cultures containing salts and pectin (1 % w/v) and 1) 0.05 M citrate, pH 5.0; 2) 0.005 M citrate, pH 5.0; 3) unbuffered. The cultures were grown for 5 d on a rotary incubator (25°C, 150 rpm). At the end of this period the filtrates were assayed for pH, PG activity and citrate concentration by the pentabromoacetone conversion procedure followed by titration of liberated iodine with sodium thiosulphate (Hargreaves, Abrahams, Vickery, 1951; Williams, 1979).

Growth was better in the presence of citrate and from the results, which have been adjusted to allow for experimental error, it is clear that a proportion has been utilised. In a separate experiment VAA was shown to be capable of growing on 100 ml 0.05 M citrate as sole carbon source (final culture dry weight, 35 mg after 7 d). However, in keeping with many other microbial systems citrate failed to cause CR (Paigen & Williams, 1970). Even at 0.05 M PG was produced at high levels whereas in the presence of glucose at similar concentrations PG would only be synthesised at basal levels.

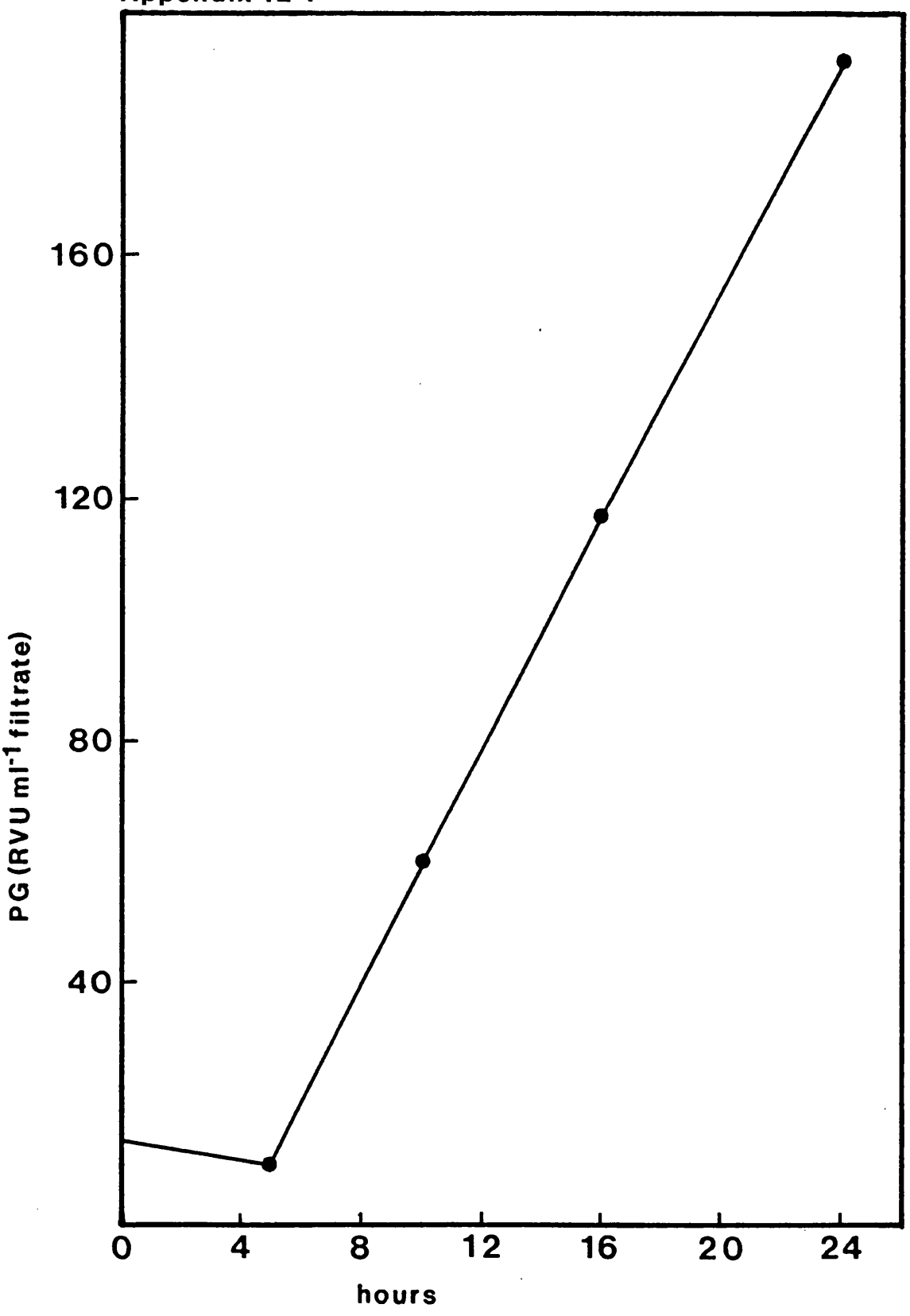
Cont..

Appendix 12 cont...

To confirm that PG was actually induced in the presence of citrate, glucose-free established cultures of VAA were resuspended in pectin (1 % w/v) containing citrate buffer (pH 5.0, 0.05 M).

PG induction occurred after a delay of only 5 h on addition of pectin Appendix 12.1.). The presence of citrate clearly did not repress the synthesis of PG.

Appendix 12·1



Appendix 13. Manifestation of symptoms on the lower 8 leaves, on 7 replicate tomato plants infected with VAA pectinase-deficient mutants; experiment 1 (see Table 31 for statistical analysis of data).

Epinasty

	Days after inoculation				
Strain	13	15	17	20	23
+Type 1	4	8	8	8	8
+Type 2	3	5	7	8	8
+Type 3	4	4	5	6	7
+Type 4	3	8	5	8	8
+Type 5	0	7	8	8	8
+Type 6	7	7	7	7	8
+Type 7	3	6	7	7	7
C23 1	0	0	1	1	2
C23 2	0	1	1	1	1
C23 3	1	1	1	2	2
C23 4	3	8	8	8	8
C23 5	2	2	3	5	6
C23 6	3	3	4	5	5
C23 7	3	3	4	4	6
24d 1	3	4	7	8	8
24d 2	0	1	1	3	3
24d 3	0	2	6	8	8
24d 4	0	2	6	8	8
24d 5	3	3	6	8	8
24d 6	4	4	4	4	4
24d 7	2	3	6	6	6
34i 1	3	4	6	7	7
34i 2	3	3	7	7	7
34i 3	1	1	4	4	4
34i 4	3	3	6	8	8
34i 5	2	3	5	5	5
34i 6	1	1	4	4	5
34i 7	2	4	8	8	8
111b 1	2	3	3	3	3
111b 2	3	8	8	8	8
111b 3	7	8	8	8	8
111b 4	7	8	8	8	8
111b 5	5	5	5	7	7
111b 6	3	7	7	7	7
111b 7	8	8	8	8	8
Cont. 1	0	0	0	2	2
Cont. 2	0	0	1	2	2
Cont. 3	2	2	2	2	2
Cont. 4	2	2	2	2	2
Cont. 5	0	0	0	0	0
Cont. 6	0	0	1	1	1
Cont. 7	0	0	0	0	0

Chlorosis

Strain	Days after inoculation 13	15	17	20	23
+Type 1	0	0	2	3	4
+Type 2	0	0	2	2	2
+Type 3	0	0	4	4	4
+Type 4	0	0	2	3	5
+Type 5	0	0	1	1	2
+Type 6	0	0	2	2	2
+Type 7	0	0	0	1	3
C23 1	0	0	0	1	1
C23 2	0	0	0	0	1
C23 3	0	0	0	1	1
C23 4	0	0	1	2	2
C23 5	0	0	2	3	3
C23 6	0	0	1	1	1
C23 7	0	0	0	1	1
24d 1	0	0	2	2	2
24d 2	0	0	0	2	4
24d 3	0	0	0	1	2
24d 4	0	0	0	1	2
24d 5	0	0	0	2	3
24d 6	0	0	0	0	1
24d 7	0	0	0	1	2
34i 1	0	0	2	2	2
34i 2	0	0	0	1	2
34i 3	0	0	0	1	2
34i 4	0	0	1	2	4
34i 5	0	0	0	1	3
34i 6	0	0	0	1	3
34i 7	0	0	2	3	3
111b 1	0	0	2	2	2
111b 2	0	0	1	2	3
111b 3	0	0	1	2	3
111b 4	0	0	2	5	7
111b 5	0	0	1	2	2
111b 6	0	0	2	2	4
111b 7	0	0	2	3	2
Cont. 1	0	0	0	0	0
Cont. 2	0	0	0	0	0
Cont. 3	0	0	0	0	1
Cont. 4	0	0	0	0	0
Cont. 5	0	0	0	0	0
Cont. 6	0	0	1	1	1
Cont. 7	0	0	0	1	1

Wilting

Strain	Days after inoculation 17	20	23	25	26
+Type 1	0	0	7	8	8
+Type 2	0	0	0	0	0
+Type 3	0	0	4	8	8
+Type 4	0	0	4	8	8
+Type 5	0	0	3	7	8
+Type 6	0	0	8	8	8
+Type 7	0	0	5	8	8
C23 1	0	0	1	4	4
C23 2	0	0	0	0	0
C23 3	0	0	0	0	0
C23 4	0	0	1	2	2
C23 5	0	0	2	2	3
C23 6	0	0	2	5	6
C23 7	0	0	0	0	0
24d 1	0	0	3	5	8
24d 2	0	0	0	2	6
24d 3	0	0	0	6	8
24d 4	0	0	1	8	8
24d 5	0	0	1	7	8
24d 6	0	0	0	0	0
24d 7	0	0	2	8	8
34i 1	0	0	3	8	8
34i 2	0	0	5	8	8
34i 3	0	0	2	6	8
34i 4	0	0	3	7	8
34i 5	0	0	2	6	8
34i 6	0	0	2	7	8
34i 7	0	0	4	8	8
111b 1	0	0	0	2	6
111b 2	0	0	3	8	8
111b 3	0	0	8	8	8
111b 4	0	0	8	8	8
111b 5	0	0	0	0	2
111b 6	0	0	4	8	8
111b 7	0	0	7	8	8
Cont. 1	0	0	0	0	0
Cont. 2	0	0	0	0	0
Cont. 3	0	0	0	0	0
Cont. 4	0	0	0	0	0
Cont. 5	0	0	0	0	0
Cont. 6	0	0	0	0	0
Cont. 7	0	0	0	0	0

7 replicate 6 week old plants were each inoculated with 50 ml 1 x 10^7 spores ml^{-1} from 3 d glucose salts cultures on 9.6.86. Weather conditions were cloudy, cold and wet. The plants were not stressed until the 23rd day when the weather turned hot and dry. Greenhouse temperature remained at *c* 20°C throughout experiment. (see Results and Discussion 3.3.).

Appendix 14. Manifestation of symptoms on the lower 8 leaves on 10 replicate tomato plants infected with VAA pectinase-deficient mutants; experiment 2 (see Table 32 for statistical analysis).

Epinasty

		Days after inoculation					
Strain	11	13	14	15	16	17	18
+Type 1	1	4	4	8	8	8	8
+Type 2	1	3	7	8	8	8	8
+Type 3	0	5	7	8	8	8	8
+Type 4	4	8	8	8	8	8	8
+Type 5	1	5	8	8	8	8	8
+Type 6	5	5	8	8	8	8	8
+Type 7	1	7	8	8	8	8	8
+Type 8	0	5	8	8	8	8	8
+Type 9	2	8	8	8	8	8	8
+Type 10	1	8	8	8	8	8	8
C23 1	0	0	2	4	4	4	4
C23 2	4	6	6	6	6	6	6
C23 3	3	3	3	3	3	3	3
C23 4	1	0	2	5	5	6	7
C23 5	2	3	4	4	4	4	6
C23 6	0	0	0	0	0	0	0
C23 7	1	3	8	7	7	7	7
C23 8	2	2	3	3	3	3	3
C23 9	0	0	0	0	0	0	0
C23 10	0	0	0	0	0	0	0
24d 1	2	2	5	5	5	6	6
24d 2	3	3	6	7	7	7	8
24d 3	0	2	3	7	7	8	8
24d 4	3	4	4	8	8	8	8
24d 5	3	4	5	8	8	8	8
24d 6	1	4	6	8	8	8	8
24d 7	0	3	5	8	8	8	8
24d 8	4	5	4	8	8	8	8
24d 9	2	3	5	7	7	6	6
24d 10	1	3	4	6	6	8	8
34i 1	2	3	5	8	8	8	8
34i 2	2	6	6	6	8	8	8
34i 3	1	4	4	4	4	8	8
34i 4	0	5	8	8	8	8	8
34i 5	0	2	2	2	2	2	2
34i 6	4	8	8	8	8	8	8
34i 7	1	3	6	6	6	6	6
34i 8	5	5	8	8	8	8	8
34i 9	2	4	5	8	8	8	8
34i 10	2	4	6	6	6	8	8

cont...

Epinasty cont...

	Days after inoculation						
Strain	11	13	14	15	16	17	18
111b 1	4	6	6	7	8	8	8
111b 2	1	7	7	7	7	7	7
111b 3	0	3	7	7	7	8	8
111b 4	5	7	8	8	8	8	8
111b 5	3	7	7	7	7	8	8
111b 6	5	7	8	8	8	8	8
111b 7	1	7	7	7	7	7	7
111b 8	1	4	8	8	8	8	8
111b 9	0	8	8	8	8	8	8
111b 10	2	4	7	7	7	8	8
Cont.1	0	0	0	0	0	0	0
Cont.2	2	2	4	4	4	4	4
Cont.3	0	0	0	0	0	0	0
Cont.4	2	5	6	6	6	6	6
Cont.5	0	2	3	4	4	4	4
Cont.6	4	5	6	6	6	6	6
Cont.7	0	0	0	0	0	0	0
Cont.8	0	0	0	0	0	0	0
Cont.9	0	0	0	0	0	0	0
Cont.10	3	6	8	8	8	8	8

Chlorosis

Strain	Days after inoculation 11	13	14	15	16	17	18
+Type 1	2	2	3	3	3	3	4
+Type 2	3	2	3	3	3	3	3
+Type 3	1	2	2	2	2	3	4
+Type 4	1	3	3	3	4	4	4
+Type 5	1	3	2	2	3	4	4
+Type 6	1	2	3	3	3	3	3
+Type 7	1	3	3	3	3	3	4
+Type 8	2	3	4	3	4	4	4
+Type 9	2	4	4	3	4	4	4
+Type 10	2	3	3	3	3	3	4
C23 1	2	2	2	2	2	2	2
C23 2	0	0	0	1	1	2	2
C23 3	2	1	2	2	3	3	3
C23 4	2	1	2	2	2	3	3
C23 5	2	2	3	3	3	3	3
C23 6	0	1	1	1	1	1	1
C23 7	1	1	1	1	1	2	2
C23 8	2	2	2	2	2	2	2
C23 9	2	2	2	2	2	2	2
C23 10	2	2	2	3	3	3	3
24d 1	1	2	2	2	2	2	2
24d 2	1	1	1	1	2	2	2
24d 3	0	0	1	1	2	2	2
24d 4	1	2	2	2	2	2	2
24d 5	1	1	2	2	2	2	2
24d 6	1	1	1	1	2	2	2
24d 7	2	2	2	2	2	2	2
24d 8	0	2	2	3	3	3	3
24d 9	1	2	2	2	2	2	3
24d 10	0	0	0	0	2	2	3
34i 1	0	0	0	0	2	2	2
34i 2	1	2	2	3	3	3	3
34i 3	1	2	2	2	2	2	2
34i 4	1	2	2	2	2	2	2
34i 5	1	2	2	2	2	2	2
34i 6	0	0	0	1	2	2	2
34i 7	0	2	3	3	3	3	3
34i 8	2	2	2	2	3	4	4
34i 9	1	2	2	2	3	3	3
34i 10	2	2	2	2	2	2	2

cont...

Chlorosis cont...

	Days after inoculation						
Strain	11	13	14	15	16	17	18
111b 1	1	2	2	2	2	4	5
111b 2	2	4	3	3	3	3	4
111b 3	2	2	3	3	3	3	4
111b 4	2	3	3	3	3	5	6
111b 5	2	2	3	3	3	5	6
111b 6	1	2	2	3	3	5	5
111b 7	2	2	2	2	2	2	3
111b 8	2	3	2	2	3	3	3
111b 9	0	1	2	2	2	4	4
111b 10	2	2	4	4	4	4	4
Cont.1	1	2	2	2	3	3	3
Cont.2	1	1	1	1	1	1	1
Cont.3	0	2	2	2	2	2	2
Cont.4	2	2	3	3	3	3	3
Cont.5	2	2	2	2	2	2	2
Cont.6	1	2	2	2	2	2	2
Cont.7	1	1	1	1	1	1	1
Cont.8	1	1	1	1	1	1	1
Cont.9	2	2	2	2	2	2	2
Cont.10	1	2	2	2	2	2	2

Wilting

Strain	Days after inoculation 11	13	14	15	16	17	18
+Type 1	0	0	0	1	0	7	8
+Type 2	0	0	0	0	0	4	8
+Type 3	0	0	0	0	1	8	8
+Type 4	0	8	8	8	8	8	8
+Type 5	0	0	2	3	3	8	8
+Type 6	0	0	0	5	7	8	8
+Type 7	0	0	0	2	3	7	8
+Type 8	0	0	0	0	0	1	4
+Type 9	0	0	3	7	8	8	8
+Type 10	0	0	0	0	0	4	8
C23 1	0	0	0	0	2	4	2
C23 2	0	0	0	0	0	0	0
C23 3	0	0	0	0	5	3	3
C23 4	0	0	0	0	0	2	2
C23 5	0	0	0	0	0	1	1
C23 6	0	0	0	0	0	0	0
C23 7	0	0	0	0	0	0	0
C23 8	0	0	0	6	6	8	8
C23 9	0	0	0	0	0	0	0
C23 10	0	0	0	1	1	0	0
24d 1	0	0	0	0	0	2	8
24d 2	0	0	0	0	4	8	8
24d 3	0	0	0	0	8	8	8
24d 4	0	0	0	0	8	8	8
24d 5	0	0	0	0	8	8	8
24d 6	0	0	0	0	2	8	8
24d 7	0	0	0	1	2	2	7
24d 8	0	0	0	0	2	2	8
24d 9	0	0	0	3	1	8	8
24d 10	0	0	0	6	8	8	8
34i 1	0	0	0	2	4	7	8
34i 2	0	0	0	3	8	8	8
34i 3	0	0	0	0	0	6	8
34i 4	0	0	0	0	8	8	8
34i 5	0	0	0	0	2	3	4
34i 6	0	0	0	0	0	6	8
34i 7	0	0	0	0	0	4	8
34i 8	0	0	8	8	8	8	8
34i 9	0	0	0	0	8	8	8
34i 10	0	0	0	0	3	8	8

cont...

Wilting cont...

	Days after inoculation						
Strain	11	13	14	15	16	17	18
111b 1	0	0	0	2	2	8	8
111b 2	0	0	0	0	3	3	8
111b 3	0	0	0	0	0	6	8
111b 4	0	0	4	8	8	8	8
111b 5	0	0	0	3	5	8	8
111b 6	0	0	0	8	8	8	8
111b 7	0	0	0	0	0	2	2
111b 8	0	0	0	0	3	8	8
111b 9	0	0	0	0	0	8	8
111b 10	0	2	0	0	7	7	7
Cont.1	0	0	0	0	0	0	0
Cont 2	0	0	0	0	0	0	0
Cont.3	0	0	0	0	0	0	0
Cont.4	0	0	0	0	0	0	0
Cont.5	0	0	0	0	0	0	0
Cont.6	0	0	0	0	0	0	0
Cont.7	0	0	0	0	0	0	0
Cont.8	0	0	0	0	0	0	0
Cont.9	0	0	0	0	0	0	0
Cont.10	0	0	0	0	0	0	0

Ten replicate 6 week old plants were each inoculated with 50 ml 1 x 10^7 spores ml^{-1} from 3 d glucose salts cultures on 9.6.86. The first wilt symptoms appeared at the beginning of a period of very warm sunny weather on 23.6.86. The bright sunshine continued until the end of the experiment. (see Results and Discussion Section 3.3.). Figures in the Appendix correspond to the number of leaves on each plant showing a symptom.

References

Adelberg, E.A., Mandel, M. & Chen, G.C.C. (1965).
Optimal conditions for mutagenesis by N-methyl-N'-nitro-N-nitrosoguanidine in *Escherichia coli*. *Biochem. Biosphys. Res. Comm.* 18, 788-795.

Aist, J.R. (1976).
Cytology of penetration and infection. In *Encyclopedia of Plant Physiology* Vol. 4, eds R. Heitefuss and P.H. Williams, pp 198-221. Springer Verlag: New York.

Aist, J.R. (1983).
A survey of plant diseases caused by fungi that attack cuticularised plant surfaces. In *Infection Processes of Fungi*, eds D.W. Roberts & J.R. Aist, pp 13-15. Conference report, Bellagio conference: The Rockefeller foundation.

Al-Aidroos, K. & Seifert, A.M. (1980).
Polysaccharide and protein degradation, germination, and virulence against mosquitoes in the entomopathogenic fungus *Metarhizium anisopliae*. *J. Invertbr. Pathol.* 36, 29-34.

Albersheim, P. (1959).
Instability of pectin in neutral solutions. *Biochem. Biophys. Res. Comm.* 1, 235-256.

Albersheim, P. (1975).
The wall of growing plant cells. *Scientific American* 232, 80-95.

Albersheim, P. (1976).
The primary cell wall. In *Plant Biochemistry*, eds J. Bonner & J.E. Warner, pp 225-173. New York: Academic Press.

Albersheim, P. & Anderson, A. (1971).
Host-pathogen interactions. III. Proteins from plant cell walls inhibit polygalacturonase secreted by plant pathogens. *Proc. Natl. Acad. Sci. U.S.A.* 68, 1815-1819.

Albersheim, P. & Killias, V. (1962).
Studies relating to the purification and properties of pectin transeliminase. *Arch. Biochem. Biophys.* 97, 105-115.

Albersheim, P. & Killias, V. (1963).
Biochemistry of the cell wall in relation to infection processes. *Ann. Rev. Phytopathology* 7, 171-194.

Albersheim, P. Neukom, H. & Deuel, H. (1960a).
Splitting of pectin chains in neutral solutions. *Arch. Biochem. Biophys.* 90, 46-51.

Albersheim, P. Neukom, H. & Deuel, H. (1960b).
über die Bildung von ungersättigten Abbauprodukten durch einpektinabbavendes Enzym. *Helv. Chim. Acta.* 43, 1422-1426.

Almengor-Hecht, M.L. & Bull, A.T. (1978).
Continuous flow enrichment of a strain of *Erwinia carotovora* having specificity for methylated pectin. *Arch. Biochem. Biophys.* 90, 46.

Andrews, P. (1964).
Estimation of the molecular weights of proteins by Sephadex gel filtration. *Biochem. J.* **91**, 222-233.

Andro, T., Chambost, J., Kotoujansky, A., Cattaneo, J., Bertheau, Y., Barras, F., Van Gijsegem, F., Coleno, A. (1984).
Mutants of *Erwinia chrysanthemi* defective in secretion of pectinase and cellulase. *J. Bacteriol.* **160**, 1199-1203.

Arinze, A.E., (1985).
The adsorbtion of a polygalacturonase isozyme of *Botrydiplodia theobromae* on plant tissues and the implication on the pathogenicity of the fungus. *Phytopathol. Z.* **114**, 13-20.

Aspinall, G.O., (1980).
Chemistry of cell wall polysaccharides. In *The Biochemistry of Plants*, vol. 3, *Carbohydrates-Structure and Function*, ed J. Preiss, pp 473-500. New York: Academic Press.

Ayres, W.A., Papavizas, G.C. & Diem, A.F. (1966).
Polygalacturonate *trans*-eliminase and polygalacturonase production by *Rhizoctonia solani*. *Phytopathology* **59**, 925-930.

Ayres, P.G. & Olutiola, P.O. (1973).
Utilisation of carbohydrates by *Rhynchosporium secalis* II. Absorbtion and metabolism of glucose, galactose and galacturonic acid. *Physiologi a Pl.* **29**, 212-217.

Bailey, J.A., (1982).
Mechanisms of phytoalexin accumulation. In *Phytoalexins*, eds Bailey, J.A. & Mansfield, J.W. Blackie: Glasgow.

Baker, C.J. & Bateman, D.F. (1978).
Cutin degradation by Plant Pathogenic Fungi. *Phytopathology* 68, 1577-1584.

Baker, C.J., Whalen C.H., & Bateman, D.F. (1977).
Xylanase from *Trichoderma pseudokningii*: purification, characterisation and effects on plant cell walls and tissue, *Phytopathology* 67, 1250-1258.

Balasubramani, K.A., Deverall, B.J., & Murphy, J.V. (1971).
Changes in respiratory rate, phenoloxidase and polygalacturonase activity in and around lesions caused by *Botrytis* in leaves of *Vicia faba*. *Physiol. Plant Pathol.* **1**, 105-113.

Ballance, D.J. & Turner, G. (1985).
Pathogenicity of monoascopric cultures of crosses between races of *Colletotrichum lindemuthianum*. *Fitopatologia Braseileira* **7**, 285-294.

Bangreth, F. (1979).
Calcium-related physiolgical disorders of plants. *Ann. Rev. Phytopath.* **17**, 97-122.

Barash, I (1968).
Liberation of polygalacturonase during spore germination by *Geotrichum candidum*. *Phytopathology* 58, 1364-1371.

Bary, A. de, (1886).
über einige *Sclerotinien* und Scerltienkrankheiten. *Botan Z.* **44**, 377-379.

Basham, H.G. & Bateman, D.F. (1975a).
Killing of plant cells by pectic enzymes: the lack of direct injurous interaction between pectic enzymes or their soluble reaction products and plant cells. *Phytopathology* 65, 141-153.

Basham, H.G. & Bateman, D.F. (1975b).
Relationship of cell death in plant tissue treated with a homogenous *endo*-pectate lyase to cell wall degradation. *Physiol. Plant Pathol.* 5, 249-262.

Bateman, D.F. (1966).
Hydrolytic and *trans*eliminative degradation of pectic substances by extracellular enzymes of *Fusarium solani f. sp.phaseoli*. *Phytopathology* 56, 238-244.

Bateman, D.F. (1976).
Plant cell wall hydrolysis by plant pathogens. In *Biochemical Aspects of Plant-Parasite Relationships*, eds Friend, J. & Threlfall, D.R., pp 79-103. Academic Press: London.

Bateman, D.F. & Basham, H.G. (1976).
Degradation of plant cell walls and membranes by microbial enzymes. In *Encyclopedia of Plant Physiology*, Vol 4, eds R. Heitefuss & P.H. Williams, pp 316-355. Springer Verlag: New York.

Bateman, D.F. & Millar, R.L. (1966).
Pectic enzymes in tissue degradation. *Ann. Rev. Phytopathology* 4, 119-146.

Batista, U.G. & Chaves, G.M. (1982).
Pathogenicity of monoascoporic cultures of crosses between races of *Colletotrichum lindemuthianum*. *Fitopatologica Brasileira* 7, 285-294.

Bauer W.D., Talmadge, K.W., Keegstra, K., Albersheim, P. (1973).
The structure of plant cell walls II. The hemicellulose of the walls of suspension-cultured sycamore cells. *Pl. Physiol.* 51, 174-187.

Beckman, C.H. (1969).
The mechanisms of gel formation by swelling of simulated plant cell wall membranes and perforation plates of banana root vessels. *Phytopathology* 59, 837-843.

Beckman, C.H. & Halmos, S. (1962).
Relation of vascular occluding reactions in banana roots to pathogenicity of root-invading fungi. *Phytopathology* 52, 893-897.

Beckman, C.H. & Talboys, P.W. (1981).
Anatomy of reistance. In *Fungal Wilt Diseases of Plants*, eds Mace, M.E., Bell, A.A. & Beckman, C.H., pp 427-521. Academic Press.

Bertheau, Y., Madgidi-Hervan, E., Kotoujansky, A., Nguyen-The, C., Andro, T. & Coleno, A. (1984).
Detection of depolymerase isoenzymes after electrophoresis or electrophocusing, or in titration curves. *Anal. Biochem.* 139, 383-389.

Bishop, C.D. & Cooper, R.M. (1983a).
An ultrastructural study of root invasion in three vascular wilt diseases. *Physiol. Plant Pathol.* 22, 15-27.

Bishop, C.D. & Cooper, R.M. (1983b).
An ultrastructural study of vascular colonisation in three vascular wilt diseases I. Colonisation of susceptible cultivars. *Physiol. Plant Pathol.* 23, 323-343.

Botstein, D. & Shortle, D. (1985).
Strategies and applications of *in vitro* mutagenesis. *Science* 229, 1193-1201.

Brillouet, B. & Carré, J.M. (1983).
Composition of cell walls from *Pisum sativum, Vicia faba* and *Glycine max. Phytochemistry* 22, 521-540.

Brown, D.E. (1979).
Technology of microbial polysaccharase production. In *Microbial Polysaccharides and Polysaccharidases*, eds Berkeley, R.C.W., Gooday, G.W., Ellwood, D.C., pp 297-326. Academic Press.

Brown, W. (1915).
Studies in the physiology of parasitism I. The action of *Botrytis cinerea*. Ann. Bot. 29, 313-348.

Burdon, J.J. & Marshall, D.R. (1983).
The use of isozymes in plant disease research. In *Isozymes in Plant Genetics and Breeding*, eds Tanksley, A. & Orton, D. Elsevier.

Byrde, R.J.W. & Archer, S.A. (1977).
Host inhibition or modification of extracellular enzymes of pathogens. In *Cell Wall Biochemistry Related to Specificity in Host-Plant Pathogen Interactions*, eds Solheim, B. & Raa, J., pp 213-245. Universitetsforlaget: Oslo.

Byrde, R.J.W., Fielding, A.H. & Williams, A.H. (1960).
The role of oxidised polyphenols in the varietal resistance of apples to brown rot. In *Phenolics in Plants in Health and Disease*, ed Pridham, pp 95-99. J.B. Pergamon Press: London.

Byrde, R.J.W. & Willetts, A.J. (1977).
The Brown Rot Fungi of Fruit: Their Biology and Control, Pergamon Press: Oxford.

Carpita, N., Sabularse, D., Montezinos, D. & Delmer, D.P. (1979).
Determination of the pore size of cell walls of living cells. *Science* 205, 1144-1147.

Cervone, F., Scala, A., Forresti, M., Cacace M.G. & Novellio C. (1977).
*Endo*polygalacturonase from *Rhizoctaria fragariae*. Purification and characteristics of two isoenzymes. *Biochim. Biophys. Acta* 482, 379-385.

Cervone, F., Scala, A. & Scala, F. (1978).
Polgalacturonase from *Rhizoctonia fragiriae*. Purification and characterisation of two isoenzymes. *Physiol. Plant Pathol.* 12, 19-26.

Chang, P.L.Y. & Trevithick, J.R. (1974).
How important is secretion of enzymes through apical cell walls of fungi? *Arch. Microbiol.* **101**, 281-293.

Chatterjee, A.K, Buchanan, G.E., Behrens, M.K. & Starr, M.P. (1979).
Synthesis and excretion of polygalacturonic acid *trans*-eliminase in *Erwinia*, Yersinia, and *Klebsiella* species. *Can. J. Microbiol.* **25**, 25-102.

Chatterjee, A.K. & Starr, M.P. (1977).
Donor strains of the soft-rot bacterium *Erwinia chrysanthemi* and conjugational transfer of the pectolytic capactiy. *J. Bacteriol.* **132**, 862-869.

Chesson, A., (1980).
A review. Maceration in relation to the post-harvest handling and processing of plant material. *J. Appl. Bacteriol.* **48**, 1-45.

Clarkson, J.C. (1982).
Genetical studies of Verticillium albo-atrum (R. & B.) causing vascular wilt disease in hop (*Humulus lupulus*). Ph.D. Thesis, University of London.

Clarkson, J.C. & Heale, J.B. (1985b).
Heterokaryon compatibility and genetic recombination within a host plant between hop isolates of *Verticillium albo-atrum*. *Plant Pathology* **34**, 129-138.

Cohen, B.L., (1973).
The neutral and alkaline proteases of *Aspergillus nidulans*. *J. Gen. Microbiol.* **77**, 521-528.

Collmer, A. & Bateman, D.F. (1981).
Impaired induction and self-catabolite repression of extracellular pectate lyase in *Erwinia chrysanthemi* mutants deficient in oligogalacturonide lyase. *Proc. Natl. Acad. Sci. U.S.A.* **78**, 3920-3924.

Collmer, A. & Bateman, D.F. (1982).
Regulation of extracellular pectate lyase in *Erwinia chrysanthemi*: evidence that reaction products of pectate lyase and *exo*-poly-α-D-galacturonidase mediate induction on D-galacturonan. *Physiol. Plant. Pathol.* **21**, 127-139.

Collmer, A., Whalen, C.H., Beer, S.V. & Bateman, D.F. (1982a).
An *exo*-poly-α-D-galacturonidase implicated in the regulation of extracellular pectate lyase production in *Erwinia chrysanthemi*. *J. Bacteriol.* **149**, 626-634.

Collmer, A., Berman, P. & Mount, M.S. (1982b).
Pectate lyase regulation and bacterial soft rot pathogenesis. In *Phytopathogenic Prokaryotes*, eds Mount, M.S. & Lacey, G.H., pp 395-442.

Collmer, A. & Keen, N.T. (1986).
The Role of Pectic Enzymes in Plant Pathogenesis. (In preparation).

Collmer, A., Schoedel, C., Roeder, D.L., Reid J.L. & Rissler, J.F. (1985).
Molecular cloning in *Escherichia coli* of *Erwinia chrysanthemi* genes encoding multiple forms of pectate lyase. *J. Bacteriol.* **161**, 913-920.

Cooke, R.C. & Whipps, J.M. (1980).
The evolution of nutrition in fungi parasitic on terrestial plants. *Biol. Rev.* 55, 341-362.

Cooper, R.M. (1974).
Cell wall degrading enzymes in vascular wilt diseases. *Ph.D. thesis, University of London.*

Cooper, R.M. (1977).
Regulation of synthesis of cell wall-degrading enzymes of plant pathogens. In *Cell Wall Biochemistry Related to Specificity in Host-Plant Pathogen Interactions*, eds Solheim, B. & Raa, J., pp 163-211. Universitetsforlaget: Oslo.

Cooper, R.M. (1981).
Pathogen induced changes in host ultrastructure. In *Plant Disease Control: Resistance and Susceptibility*, eds Staples, R.C. & Toenniesen, G.H., pp 105-142. Wiley: New York.

Cooper, R.M. (1983).
The mechanisms and significance of enzymic degradation of host cell walls. In *Biochemical Plant Pathology*, ed Callow, J.A., pp 101-137. Wiley.

Cooper, R.M. (1984).
The role of cell wall-degrading enzymes in infection and damage. In *Plant Diseases: Infection, Damage and Loss*, eds Wood, R.K.S. & Jellis, G.J., pp 13-28. Blackwells: Oxford.

Cooper, R.M. (1986a).
The use of mutants in exploring extracellular depolymerases as determinants of pathogenicity. In *Genetics and Plant Pathogenesis*, eds Day, P.R. & Jellis, G.J. (In preparation). Blackwells.

Cooper, R.M. (1986b).
Host cell wall degradation by microbial plant pathogens. Biological Reviews (in press).

Cooper, R.M., Rankin, B. & Wood, R.K.S. (1978).
Cell wall-degrading enzymes of vascular wilt fungi. II. Properties and modes of action of polysaccharidase of *Verticillium albo-atrum* and *Fusarium oxysporum f. sp. lycopersici*. *Physiol. Plant Pathol.* 13, 101-134.

Cooper, R.M., Whardman, P.A. & Skelton, J.E.M. (1981).
The influence of cell walls from host and non-host plants on the production and activity of polygalacturonide-degrading enzymes from fungal pathogens. *Physiol. Plant Pathol.* 18, 229-255.

Cooper, R.M. & Wood, R.K.S. (1973).
Induction of synthesis of extracellular cell wall-degrading enzymes in vascular wilt fungi. *Nature* 246, 309-311.

Cooper, R.M. & Wood, R.K.S. (1975).
Regulation of synthesis of cell wall-degrading enzymes by *Verticillium albo-atrum* and *Fusarium oxysporum f. sp. lycopersici*. *Physiol. Plant Pathol.* 5, 135-156.

Cooper, R.M. & Wood, R.K.S. (1980).
Cell wall-degrading enzymes of vascular wilt fungi. III. Possible involvement of *endo*pectin lyase in *Verticillium* wilt of tomato. *Physiol. Plant Pathol.* 16, 285-300.

Cowling, E.B. (1975).
Physical and chemical constraints in the hydrolysis of cellulose and lignocellulosic materials, in *Biochemical and Bioengineering Symposium 5*, ed Wilkie, C.R., pp 163-181. Wiley: New York.

Cromburgghe, B., de, Chen, B., Anderson, W., Nissley, P., Gottesman, M. & Pastan, I. (1971).
Lac DNA, RNA polymerase and cyclic AMP receptor protein, cyclic AMP. *lac* repressor and inducer are the essential elements for controlled *lac* transcription. *Nature*, 231, 139-142.

Cruickshank, R.H. (1983a).
Distinction between *Sclerotinia* species by their pectic zymgramms. *Trans. Br. mycol. Soc.* 80, 117-119.

Cruickshank, R.H. (1983b).
Pectic enzymes and the taxonomy of *Botrytis*. *Fourth Congress of Plant Pathology* (Melbourne), abstract, 708.

Cruickshank, R.H. & Wade, G.C. (1980).
Detection of pectic enzymes in pectin-acrylamide gels. *Anal. Biochem.* 107, 177-181.

Daniels, M.J., Barber, C.E., Turner, P.C., Cleary & Sawycyz M.K. (1984).
Isolation of mutants of *Xanthomonas campestris pv. campestris* showing altered pathogenicity. *J. Gen. Microbiol.* 130, 2447-2455.

Daniels, M.J, Barber, C.E., Turner, P.C., Sawzyc, M.K., Byrde, R.J.W. & Fielding, A.H. (1984).
Cloning of genes involved in pathogenicity of *Xanthomonas campestris pv. campestris* using the broad host range cosmid pL AFR1. *EMBO J.* 3, 3323-3328.

Darvill, A.G., McNeil, M., Albersheim, P. & Delmer, D.P. (1980a).
The primary walls of flowereing plants. In *The Biochemistry of Plants*, vol. 1, pp 91-162, ed Tolbert, N.E.. Academic Press: New York.

Davis, K.R., Lyon, G.D., Darvill, A.G. & Albersheim, P. (1984).
Endopolygalacturonic acid lyase from *Erwinia carotovora* elicits phytoalexin accumulation by releasing plant cell wall fragments. *Plant Physiol.* 74, 52-60.

Dawson, R.M.C., Elliot, D.C., Elliot, W.H. & Jones, K.M., eds, (1969).
Data for Biochemical Research, 2nd edition. Oxford University Press.

Défago, G. & Kern, H. (1983).
Induction of *Fusarium solani* mutants insensitive to tomatine, their pathogenicity and aggressiveness to tomato fruits and pea plants. *Physiol. Plant Pathol.* 22, 29-37.

Défago, G., Kern, H. & Sedler, L. (1983).
Genetic analysis of tomatine insensitivity, sterol content and pathogenicity for green tomato fruits in mutants of *Fusarium solani*. *Physiol. Plant Pathol.* 22, 39-43.

Demain, A.L. (1971).
Overproduction of microbial metabolites and enzymes due to alteration of regulation. *Advances in Biochemical Engineering* 1, 113-142.

Demain, A.L. (1972).
Theoretical and applied aspects of enzyme regulation and biosynthesis in microbial cells. *Biotechnology and Bioengineering Symposium* 3, 21-32

Dickman, M.B. & Patil, S.S. (1986).
Cutinase deficient mutants of *Colletotrichum gloeosphorioides* are non-pathogenic to papaya fruit. *Physiol. Molec. Plant Pathol.* 28, 235-242.

Dickman, M.B., Patil, S.S. & Kolattukudy, P.E. (1983).
Purification, characterisation and role in infection of an extracellular cutinolytic enzyme from *Colletotrichum gloeosphorioides* on *Carica papava*. *Physiol. Plant Pathol.* 20, 333-347.

Di Lenna, P. & Fielding, A.H. (1983).
Multiple forms of polygalacturonase in apple and carrot infected by isolates of *Botrytis cinerea*. *J. Gen. Microbiol.* 129, 3015-3018.

Dimond, A.E. (1970).
Biophysics and biochemistry of the vascular wilt syndrome. *Ann. Rev. Phytopathol.* 8, 301-322.

Dingle, J., Reid, W.W. & Solomons, G.L. (1953).
The enzymic degradation of pectin and other polysaccarides. II. Application of the cup plate assay to the estimation of enzymes. *J. Sci. Food. Agric.* 4, 149-155.

Dixon, G.R. & Pegg, G.F. (1972).
Changes in amino acid content of tomato xylem sap following infection with strains of *Verticillium albo-atrum*. *Ann. Bot.* 36, 147-154.

Dodman, A.B. (1979).
How the defenses are breached. In *Plant Disease-An Advanced Treatise*, vol 4, *How Pathogens Induce Disease*, eds Horsfall, J.G. & Cowling, E.B., pp 135-153

Drake, J.W. & Baltz, R.H. (1976).
The biochemistry of mutagenesis. *Ann. Rev. Biochem.* 45, 11-37.

Easton, C.J. & Rossall, S. (1985).
The production of certain cell wall-degrading enzymes by *Leptosphaeria maculans* in culture and in stem canker lesions of oilseed rape. *Physiol. Plant Pathol.* 26, 185-197.

Enari, T-M. (1983).
Microbial cellulases. In *Microbila Enzymes and Biotechnology*, ed Fogarty, W.M., pp 183-233. Applied Science Publishers Ltd.

English, P.D., Jurale, J.B. & Albersheim, P. (1971).
Host-pathogen interactions. II. Parameters affecting polysaccharide-degrading enzyme secretion by *Colletotrichum lindemuthianum* grown in culture. *Plant Physiol.* **47**, 1-6.

English, P.D., Maglothin, A., Keegstra, K. & Albersheim, P. (1972).
A cell wall-degrading *endo*-PG secreted by *Colletotrichum lindemuthianum. Plant Physiol.* **49**, 293-297.

Eriksson, K.E. (1977).
Degradation of wood cell walls by the rot fungus *Sporotrichum pulverultenum*-enzyme mechanisms. In *Cell Wall Biochemistry Related to Specificity in Host-Plant Pathogen Interactions*, eds Solheim, B. & Raa, J., pp 71-83. Universitetsforlaget, Oslo.

Eriksson, K.E. & Goodell, E.W. (1974).
Pleitropic mutants of the wood-rotting fungus *Polyporus adusta* lacking cellulase, mannanase and xylanase. *Can J. Microbiol.* **20**, 371-378.

Eriksson, K.E. & Winnell, M. (1968).
Purification and characterisation of a fungal β-mannanase. *Acta. Chem. Scand.* **22**, 1924-1934.

Esser, K. & Kuenen, R. (1967).
Genetics of Fungi. Springer Verlag: New York.

Eveleigh, D.E. & Montenecourt, B.S. (1979).
Increasing yields of extracellular enzymes. *Advances in Applied Microbiology* **25**, 57-74.

Faith, W.T., Neubeck, C.E. & Reese, E.T. (1971).
Production and application of enzymes. *Adv. Biochem. Eng.* **1**, 77-111.

Fantes, P.A. & Roberts, C.F. (1973).
β-galactosidase activity and lactose utilisationin *Aspergillus nidulans. J. Gen. Microbiol.* **77**, 471-486.

Fergusson, M.W. & Chatterjee, A.K. (1981).
Characterisation of *Erwinia chrysanthemi* mutants deficient in synthesis of polygalacturonic acid trans-eliminase. *Phytopathology* **71**, 216, (Abst.).

Fernando, G., Zimmermann, W. & Kolattukudy, P.E. (1984).
Suberin-grown *Fusarium solani* f. sp. pisi generates a cutinase-like esterase which depolymerises the aliphatic components of suberin. *Physiol. Plant Pathol.* **24**, 143-155.

Ferro-Novick, S., Novick, P., Field, C. & Schekman, R. (1983).
Secretory mutants that block the formation of active cell surface enzymes. *J. Cell Biol.* **98**, 35-43.

Ferro-Novick, S., Hansen, W., Schauer, I. & Schekman, R. (1984).
Genes required for completion of import of proteins into the endoplasmic reticulum in yeast. *J. Cell Biol.* **98**, 44-53.

Fielding, A.H. (1981).
Natural inhibitors of fungal polygalacturonases in infected fruit tissues. *J. Gen. Microbiol.* 123, 377-381.

Fleming, I.D. & Pelger, H.F. (1963).
The determination of glucose in the presence of maltose and isomaltose by a stable specific enzymic reagent. *Analyst, London* 88, 967-968.

Fogarty, W.M. & Kelly, C.T. (1983).
Pectic enzymes. In *Microbial Enzymes and Biotechnology*, ed Fogarty, W.M., pp 131-182. Applied Science Publishers Ltd.

Fox, R.T.V., Manners, J.G. & Myers (1972).
Ultrastructure of tissue disintegration and host reactions in potato tubers infected with *Erwinia carotovora* var. *atroseptica*. *Potato Res.* 15, 130-145.

Friend, J. (1976).
Lignification in infected tissues. In *Biochemical aspects of plant-parasite relationships*, eds Friend, J. & Threlfall, D.R., pp 291-304. Academic Press: London and New York.

Friend, J. (1980).
Plant phenolics, lignification and plant disease. In *Progress in Phytochemistry*, eds Reinhold, L., Harborne, J.B. & Swain, T., pp 197-261.

Fry, S.C., Darvill, A.G. & Albersheim, P. (1983).
In interactions between nitrogen and growth regulators in the control of plant development. British Plant Regulator Group. Monograph 9., ed Jackson, M.B., pp 33-43.

Garibaldi, A. & Bateman, D.F. (1971).
Pectic enzymes produced by *Erwinia chrysanthemi* and their effects on plant tissue. *Physiol. Plant Pathol.* 1, 25-40.

Gianazza, E. & Rignetti, P.G. (1980).
Facts and artifacts in isoelectric focusing. In *Electrophoresis '79: advanced methods, biochemical and clinical applications; Proceedings of the Second International Conference on Electrophoresis*, ed Radola, B.J. pp 129-140.

Goodenough, U. (1978).
Genetics, 2nd edition. Holt Saunders: Philadelphia.

Gomori, G. (1950).
Methods of study for tissue lipase. In *'Menstruation and its disorders'*, Engle, E.T. pp 52-61. Charles C. Thomas: USA.

Gordon, A.H., Morris, E.R., Rees, D.A., Smith, P.J.C., & Thorn, D. (1977).
Polysaccharides and associated components of mesophyll cell walls prepared from grasses. *Carbohydr. Res.* 57, 235-248.

Hall, J.A. & Wood, R.K.S. (1973).
The killing of plant cells by pectolytic enzymes. In *Fungal Pathogenicity and the Plant's Response*, eds Byrde, R.J.W. & Cutting, C.V., pp 19-38. Academic Press: London.

Hancock, J.G. (1966).
Degradation of pectic substances associated with pathogenesis by *Sclerotinia sclerotiorum* in sunflower and tomato stems. *Phytopathology* 56, 975-979.

Hancock, J.G. (1968).
Degradation of pectic substances during pathogenesis by *Fusarium solani f. sp. cucurbitae*. *Phytopathology* 58, 62-69.

Hancock, J.G. & Lorbeer, J.W. (1963).
Pathogenesis of *Botrytis cinerea*, *B. squamosa* and *B. allii* on onion leaves. *Phytopathology* 53, 669-673.

Hancock, J.G. Millar, R.L. & Lorbeer, J.W. (1964a).
Pectolytic and cellulolytic enzymes produced by *Botrytis allii*, *B. cinerea* and *B. squamosa in vitro* and *in vivo*. *Phytopathology* 54, 928-931.

Hancock, J.G. Millar, R.L. & Lorbeer, J.W. (1964b).
Role of pectic and cellulolytic enzymes in *Botrytis* leaf blight on onion. *Phytopathology* 54, 932-935.

Hankin, L. & Anagnostakis, S.L. (1975).
The use of solid media for detection of production by fungi. *Mycologica* 67, 597-607.

Hargreaves, C.A. Abrahams, H.M. & Vickery, H.B. (1951).
Determination of citric and δ-isocitric acids. *Anal. Chem.* 23, 467-470.

Hastie, A.C. (1964).
The parasexual cycle in *Verticillium albo-atrum*. *Genetical Research* 5, 305-315.

Hastie, A.C. (1968).
Phialide analysis of mitotic recombination in *Verticillium*. *Molec. Gen. Genet.* 102, 232-240.

Hastie, A.C. & Heale, J.B. (1984).
Genetics of *Verticillium*. *Phytopathologica Mediterranea* 23, 130-162.

Hawksworth, D.L. & Talboys, P.W. (1970).
Verticillium dahliae. C.M.I. description of pathological fungi and bacteria no. 256.

Heale, J.B. & Gupta, D.P. (1972).
Mechanism of vascular wilting induced by *Verticillium albo-atrum*. *Trans. Br mycol. Soc.* 58, 19-28.

Heath, M.C. (1985).
Resistance methods in host and non-host plants. In *Natural Antimicrobial Systems*. Bath University Press.

Hilu, H.M. & Bever, W.M. (1957).
Inoculation, oversummering and suscept-pathogen relationship of *Septoria tritici* on *Triticum* species. *Phytopathology* 47, 474-480.

Hislop, E.C., Keon, J.P.R. & Fielding, A.H. (1979).
Effect of pectin lyase from *Monilinia fructigena* on viability, ultrastructure and localisation of acid phosphatase of cultured apple cells. *Physiol. Plant Pathol.*, 14, 371-381.

Hoffman, R.M. & Turner, J.G. (1984).
Occurence of an *endo*polygalacturonase inhibitor in *Pisum sativum*. *Physiol. Plant Pathol.*, 24, 49-59.

Hoffman, R.M. & Wood, T.M. (in preparation).
Isolation and partial characterisation of a mutant of *Penicillium funicolosum* for the saccharification of straw.

Hooley, P., Fyfe, A.M., Maltese, C.E. & Shaw, D.S. (1982).
Duplication cycle in nuclei of germinating zoospores of *Phytophthora drechsleri* as revealed by DAPI staining. *Trans Br. mycol. Soc.* 79, 563-566.

Hollman, S. (1964).
Non-glycolytic pathways of metabolism of glucose. Academic Press: London.

Holtz, G. & Knox-Davies, P.S. (1985a).
Production of pectic enzymes by *Fusarium oxysporum f. sp. cepae* and its involvement in onion bulb rot. *Phytopath. Z.* 112, 69-80.

Holtz, G. & Knox-Davies, P.S. (1985b).
Pectic enzyme production by *Fusarium oxysporum f. sp. cepae*: induction by cell walls from different parts of onion bulbs at different growth stages. *Phytopath. Z.* 112, 81-92.

Holtz, G. & Knox-Davies, P.S. (1985c).
Natural sugars present in different parts of onion bulbs at different growth stages in relation to pectic enzyme production by *Fusarium oxysporum f. sp. cepae*. *Phytophlactica* 17, 157-161.

Holtz, G. & Knox-Davies, P.S. (1986a).
Possible involvement of apoplast sugar in endo-pectin-*trans*-eliminase synthesis and onion bulb rot caused by *Fusarium oxysporum f. sp. cepae*. *Physiol Molec. Plant Pathol.* 28, 403-410.

Holtz, G. & Knox-Davies, P.S. (1986b).
Relation between endo-pectin-*trans*-elininase and apoplast-symplast sugars in Fusarium bulb rot of onions. *Physiol Molec. Plant Pathol.* 28, 411-421.

Hoppe, H.H. & Heitefuss, R. (1974).
Permeability and membrane lipid metabolism of *Phaseolus vulgaris* infected with *Uromyces phaseoli*. III. Changes in relative concentration of lipid bound fatty acids and phosphatase activity. *Physiol. Plant Pathol.* 4, 11-24.

Horton, J.G. & Keen, N.T. (1966a).
Regulation of induced cellulase synthesis in *Pyrenochaeta terrestris* by utilisable carbon compounds. *Can. J. Microbiol* 12, 209-220.

Horton, J.G. & Keen, N.T. (1966b).
Sugar repression of *endo*-polygalacturonase and cellulase synthesis during pathogenesis by *Pyrenochaeta terrestris* as a resistance mechanism in onion pink root rot. *Phytopathology* 56, 908-916.

Howell, C.R. (1976).
Use of enzyme deficient mutants of *Verticillium dahliae* to assess the importance of pectolytic enzymes in symptom expression of *Verticillium* wilt of cotton. *Physiol. Plant Pathol.* 9, 279-283.

Howell, H.E. (1975).
Correlation of virulence with secretion *in vitro* of three wall-degrading enzymes in isolates of *Sclerotinia fructigena* obtained after mutagen treatment. *J. Gen. Microbiol.* **90**, 32-40.

Hsu, G.J. & Vaughn, R.H. (1969).
Production and catabolite repression of the constitutive polygalacturonic acid *trans*-eliminase of *Aeromonas liquefaciens*. *J. Gen Microbiol.* **98**, 172-181.

Hulme, M.A. & Stranks, D.W. (1971).
Regulation of cellulase production by *Myrothecium verrucaria* grown on cellulosic substrates. *J. Gen Microbiol.* **69**, 172-181.

Hutson, R.A. & Mansfield, J.W. (1980).
A genetical approach to the analysis of the mechanisms of pathogenicity in *Botrytis/Vicia faba* interactions. *Physiol. Plant Pathol.* **17**, 309-317.

Ingram, D.S., Sargent, J.A. & Tommerup, I.C. (1974). Structural aspects of infection by biotrophic fungi. In *Biochemical Aspects of Plant Parasite Relationships*, eds Friend, J. & Threlfall, D.R., pp 43-78. Academic Press: New York.

Jacob, F. & Monod, J. (1961).
Genetic regulatory mechanisms in the synthesis of proteins. *J. Molec. Biol.* **69**, 397-408.

Jennings, D.H. (1974).
Sugar transport in fungi: an essay. *Trans. Br. mycol. Soc.* **62**, 1-24.

Jones, P. & Ayres, P.G. (1974).
Rhyncosporium leaf blotch of barley studied during the subcuticular phase by electron microscopy. *Physiol. Plant Pathol.* **4**, 229-233.

Jones, T.M., Anderson, A.J. & Albersheim, P. (1972).
Host pathogen interactions. IV. Studies on the polysaccharide-degrading enzymes secreted by *Fusarium oxysporum f. sp. lycopersici*. *Physiol. Plant Pathol.* **2**, 153-166.

Jones, T.M. & Bateman, D.F. (1972).
Galactosidase production by *Sclerotium rolfsii*. *Ibid*, **767** (Abst.).

Kalb, V.F. & Bernlohr, R.W. (1977).
A new spectrophotometric assay for protein in cell extracts. *Anal. Biochem.* **82**, 362-371.

Karr, A.L. & Albersheim, P. (1970).
Polysaccharide-degrading enzymes are unable to attack plant cell walls without prior action by a 'wall-modifying enzyme'. *Plant Physiol.* **46**, 69-80.

Keegstra, Talmadge, K.W., Bauer, W.D. & Albersheim, P. (1973).
The structure of plant cell walls. III. A model of the walls of suspension-cultures sycamore cells based on the interconnections of the macro-molecular components. *Plant Physiol.* **51**, 188-196.

Keen, N.T., Dahlbeck, D., Staskawicz, B. & Belser, W. (1984).
Molecular cloning of pectate lyase genes from *Erwinia chrysanthemi* and their expression in *Escherichia coli*. *J. Bacteriol.* **159**, 825-831.

Keen, N.T. & Erwin, D.C. (1971).
*Endo*polygalacturonase: evidence against involvement in *Verticillium* wilt of cotton. *Phytopathology* **61**, 198-203.

Keen, N.T. & Tamaki, S. (1986).
Structure of two pectate lyase genes from *Erwinia chrysanthemi* EC16 and their high level expression in *Escherichia coli*. *J. Bacteriol.* (In press).

Keen, N.T., Williams, P.H. & Walker, J.C. (1967b).
Protease of *Pseudomonas lachrymans* in relation to cucumber angular leaf spot. *Phytopathology* **57**, 263-271.

Kelly, C.T. & Fogarty, W.M. (1978).
Production and properties of polygalacturonate lyase by a alkalophilic microorganism *Bacillus* sp. RK9. *Can. J. Microbiol.* **24**, 1164-1172.

Kelman, A. & Cowling, E.B. (1965).
Cellulase of *Pseudomonas solanacearum* in relation to pathogenesis. *Phytopathology* **55**, 148-155.

Kenning, L.A. & Hanchey, P. (1980).
Ultrastructure of lesion formation in Rhizoctonia-infected bean hypocotyls. *Phytopathology* **70**, 998-1004.

Keon, J.P.R. (1982).
The effect of pectin lyase from Monilinia fructigena on the viability and ultrastructure of suspension cultured apple cells. M.Sc. Thesis, University of Bristol.

Keon, J.P.R. (1985).
Cytological damage and cell wall modification in cultured apple cells following exposure to pectin lyase from *Monilinia fructigena*. *Physiol. Plant Pathol.* **26**, 11-29.

Keon, J.P.R., Byrde, R.J.W. & Cooper, R.M. (1986).
Microbial depolymerases in the establishment and progress of pathogenic infections. In *Plant Infecting Fungi: Establishment, Progress and Outcome of Pathogenic and Mutualistic Infection*, eds Pegg, G.P. & Ayres, P.G. (In preparation): Cambridge University press.

Kilgore, W.W & Starr, M.P. (1959).
Catabolism of galacturonic and glucuronic acids by *Erwinia carotovora*. *J. Biol. Chem.* **234**, 2227-2235.

Knee, M., Fielding, A.H., Archer, S.A. & Laborda, F. (1975).
Enzymic analysis of cell wall structure in apple fruit cortical tissue. *Phytochemistry* **14**, 2213-2222.

Koenigs, J.W. (1972).
Production of extracellular hydrogen peroxide and peroxidase by wood-rotting fungi. *Phyopathology* **62**, 100-110.

Kolattukudy, P.E. (1983).
How do pathogenic fungi break the plant cuticular barrier? In *Infection processes of fungi*, eds Roberts, D.W. & Aist, R. Bellagio conference report: The Rockefeller Foundation.

Kolattukudy, P.E. & Köller, W. (1983).
Fungal penetration of the first line defensive barriers of plants. In *Biochemical plant pathology*, ed Callow, J.A., pp 79-100. Wiley.

Köller, W., Allan, C.R. & Kolattukudy, P.E. (1982).
Role of cutinase and cell wall degrading enzymes in infection of *Pisum sativum* by *Fusarium solani f. sp. pisi*. *Physiol. Plant Pathol.* **20**, 47-60.

Kotoujansky, A., Diolez, A., Baccara, V., Bertheau, Y., Andro, T. & Coleno, A. (1985). Molecular cloning of *Erwinia chrysanthemi* pectinase and cellulase structural genes. *EMBO J.* **4**, 781-785.

Kritzman, G., Chet, I. & Gilan, D. (1981).
Spore germination and penetration of *Botrytis allii* into *Allium cepa* host. *Bot Gaz.* **142**, 151-155.

Laborda, F., Archer, S.A., Fielding, A.H. & Byrde, R,J.W. (1974).
Studies on the α-λ-arabinofuranosidase complex from *Sclerotinia fructigena* in relation to brown rot of apple. *J. Gen. Microbiol.* **81**, 151-163.

Lafitte, C., Barthé, J.P., Montillet, J.L. & Touzé, A. (1984).
Glycoprotein inhibitors of *Colletotrichum lindemuthianum endo*-polygalacturonase in near isogenic lines of *phaseolus vulgaris* resistant and susceptible to anthracnose. *Physiol. Plant Pathol.* **25**, 39-53.

Lampen, J.O. (1974).
Movement of extracellular enzymes across cell membranes. In *Transport at the Cellular Level*, eds Sleigh, M.A. & Jennings, D.H. Symposia of the Society of Experimental Biology 28, 351-374.

Lamport, D.T.A. (1980). Structure and function of plant glycoproteins. In *The Biology of Plants*, Vol 3, ed Preiss, J., pp 501-541. Academic Press: New York.

Langcake, P., Bratt, P.M. & Drysdale, R.B. (1973).
Pectin methylesterase in *Fusarium*-infected susceptible tomato plants. *Physiol. Plant Pathol.* 3, 101-106.

Lapwood, D.H. (1957).
Studies in the physiology of parasitism. XXIII. On the parasitic vigour of certain bacteria in relation to their capacity to secrete pectolytic enzymes. *Ann. Bot.* **21**, 167-184.

Last, F.T. & Hamley (1956). A local lesion technique for measuring the infectivity of *Botrytis fabae* Sard. *Ann. Appl. Biol.* **44**, 410-418.

Laurent, T.C. & Killander, J. (1964).
A theory of gel filtration and its experimental verification. *J. Chromatogr.* **14**, 317-330.

Lehninger, A.L. (1975).
Biochemistry. Worth.

Lin, T.S. & Kolattukudy, P.E. (1978).
Induction of a polyester hydrolase (cutinase) by low levels of cutin monomers in *Fusarium solani f. sp. pisi*. *J. Bacteriol.* 133, 942-951.

Lisker, N. & Retig, N. (1974).
Detection of polygalacturonase and pectin lyase in polyacrylamide gels. *J. Chromatogr.* **96**, 245-249.

Lisker, N., Katan, J., Chet, I. & Henis, Y. (1975).
Release of cell bound polygalacturonase and cellulase from mycelium of *Rhyzoctonia solani*. *Can. J. Microbiol.* **21**, 521-526.

Lumpsden, R.D. & Bateman, D.F. (1968).
Phosphatide-degrading enzymes associated with pathogenesis in *Phaseolus vulgaris* infected with *Thielaviopsis basicola*. *Phytopathology* 58, 219-227.

Magasinik, B. (1961).
Catabolite repression. *Cold Spring Harbour Symposium on Quantitative Biology* **26**, 249-256.

Magro, P., Di Lenna, P., Marciano, P. & Pallavivini, C. (1980).
Variability of polygalacuronase and protein isoelectric focusing patterns in *Botrytis cinerea* isolates. *J. Gen. Microbiol.* **120**, 105-109.

Maiti, I.B. & Kolattukudy, P.E. (1979).
Prevention of fungal infection of plants by specific inhibition of cutinase. *Science* 205, 507-508.

Mankarios, A.T. & Friend, J. (1980).
Polysaccharide-degrading enzymes of *Botrytis allii* and *Sclerotium cepivorum*. Enzyme producton in culture and the effect of the enzymes on isolated onion cell walls. *Physiol. Plant Pathol.* **17**, 49-59.

Mankarios, A.T. Jones, C.F.G., Jarvis, M.C., Threlfall, D.R. & Friend, J. (1979).
Hydrolysis of plant polysaccharides and GLC analysis of their constituent neutral sugars. *Phytochemisty* **18**, 419-422.

Mankarios, A.T., Hall, M.A., Jarvis, M.C., Threlfall, D.R. & Friend, J. (1980).
Cell wall polysachharides from onions. *Phytochemistry* **19**, 1731-1733.

Mann, B. (1962).
Role of pectic enzymes in the *Fusarium* wilt syndrome of tomato. *Trans Br. mycol. Soc.* 45, 169-178.

Mansfield, J.W. (1980).
Mechanisms of resistance to *Botrytis*. In *The Biology of Botrytis*, eds Coley-Smith, J.R., Verhoeff, K. & Jarvis, W.R., pp 181-218. Academic Press: London.

Mansfield, J.W. & Hutson, R,A. (1980).
Microscopical studies on fungal development and host responses in broad bean and tulip leaves inoculated with five species of *Botrytis*. *Physiol. Plant Pathol.* **17**, 131-143.

Mansfield, J.W. & Richardson, A. (1981).
The ultrastructure of interactions between *Botrytis* species and broad bean leaves. *Physiol. Plant Pathol.* 19, 41-48.

Marcus, L. & Schejter, A. (1983).
Single step chromatographic purification and characterisation of the *endo*polygalacturonase and pectinesterase of the fungus, *Botrytis cinerea* Pers. *Physiol. Plant Pathol.* 22, 1-13.

Masurekar, P.S., Kahgan, M.P. & Demain, A.L. (1972).
Mutagenesis and enrichment of auxotrophs in *Penicillium chrysogenum*. *Appl. Biol.* 24, 995-996.

Matta, A. & Dimond, A.E. (1963).
Symptoms of *Fusarium* wilt in relation to quantity of fungus and enzyme activity in tomato stems. *Phytopathology* 53, 574-578.

Maude, R.B. (1980).
Disease control. In *The Biology of Botrytis*, eds Coley-Smith, J.R., Verhoeff, K. & Jarvis, W.R., pp 279-308. Academic Press: London.

McClendon, J.H. (1970).
Evidence for the pectic nature of the middle lamella of potato tuber cell walls based on chromatography of macerating enzymes. *Am. J. Botany* 51, 628-633.

McCord, J.M. (1974).
Free radicals and inflammation: protection of synovial fluid by superoxide dismutase. *Science* 185, 529-531.

McDonnell, K. (1958).
Relationship of pectic enzymes and pathogenicity in the *fusarium* wilt of tomatoes. *Trans. Br. mycol. Soc.* 45, 55-62.

McKeen, W.E. (1974).
Mode of penetration of epidermal cell walls of *Vicia faba* by *Botrytis cinerea*. *Phytopathology* 64, 461-467.

McKenzie, C.R. & Williams, R.E. (1984).
Detection of cellulase and xylanase activity in isoelectric-focused gels using agar substrate gels supported on plastic film. *Can. J. Microbiol.* 30, 1522-1525.

Meevootisom, V. & Niederpruem, Y. (1970).
Control of exo-cellular proteases in Dermatophytes and especially *Tricophyton rubrum*. *Sabouradia* 17, 91-106.

Menzinger, W. (1965).
Karyologische Untersuchungen an Arten und Formen der Gattung *Botrytis* Mich. *Archiv. für Mikrobiologie* 52, 178-196.

Mercer, P.C., Wood, R.K.S. & Greenwood, A.D. (1974).
Resistance to anthracnose of French Bean. *Physiol. Plant Pathol.* 4, 195-205.

Mohan, S.B. & Ride, J.P. (1984).
Some biochemical and morphological characteristics of serotypes of *Verticillium albo-atrum* from hop. *J. Gen. Microbiol.* 130, 3203-3218.

Montenecourt, B.S. & Eveleigh, D.E. (1977a).
Semi-quantitative plate assay for determination of cellulase production by *Trichoderma viride*. *Appl. Environ. Microbiol.* 33, 178-183.

Montenecourt, B.S. & Eveleigh, D.E. (1977b).
Preparation of mutants of *Trichoderma reesei* with enhanced cellulase production. *Appl. Environ. Microbiol.* 33, 777-782.

Montenecourt, B.S. & Eveleigh, D.E. (1978).
Hyper cellulolytic mutants and their role in saccharification. In *Second Annual Fuels from Biomass Symposium*, pp 613-25, ed Schuster. New York Department of Energy.

Montenecourt, B.S. & Eveleigh, D.E. (1979).
Increasing yields of extracellular enzymes. *Adv. Appl. Microbiol.* 25, 57-74.

Montenecourt, B.S., Schamhart, D.H.J. & Eveleigh, D.E. (1979).
Mechanisms controlling the synthesis of the *Trichoderma reesei* cellulase system. In *Microbial Polysaccharides and Polysaccharases*, eds Berkeley, R.C.W., Gooday, G.W. & Ellwood, D.C., pp 327-337. Academic Press.

Montgomery, R.A.P. (1982).
In *Decomposer Basidiomycetes: Their Biolgy and Ecology*, eds Frankland, J.C., Hedger, J.N. & Swift, M.J., pp 51-65. Cambridge University Press: Cambridge.

Moran, F., Nasuno, S. & Starr, M.P. (1968).
Extracellular and intracellular polygalacturonase acid *trans*-eliminase of *Erwininia carotovora*. *Arch. Biochem. Biophys.* 123, 218-306.

Morrall, R.A.A., Duczek, L.J. & Sheard, J.W. (1972).
Variations and correlations with and between morphology, pathogenicity and pectolytic enzyme activity in *Sclerotinia* from Saskatchewan. *Can. J. Bot.* 50, 767-786.

Morris, V.J. (1986).
Gelation of polysaccharides. In *Functional Properties of Food Macromolecules*, eds Mitchell, J.R. & Ledward, D.A., pp 121-201. London & New York: Elsevier Applied Science Publishers.

Mount, M.S., Bateman, D.F. & Basham, H.G. 1970.
Induction of electrolyte loss, tissue maceration and cellular death of potato tissue by an *endo*-polygalacturonate *trans*-eliminase. *Phytopathology* 60, 924-931.

Mullen, J.M. & Bateman, D.E. (1971).
Production of an *endo*-polygalacturonate *trans*-eliminase by a potato dry-rot pathogen. *Fusarium roseum* 'Avenaceum in culture and in diseased tissue. *Physiol. Plant Pathol.* 1, 363-373.

Mussell, H.W. (1973).
Endo-polygalacturonase: evidence for the involvement in *Verticillium* wilt of cotton. *Phytopathology* 63, 62-70.

Mussell, H.W. & Green, R,J. (1970).
Host colonisation and polygalacturonase production by two tracheomycotic fungi. *Phytopathology* 60, 46-51.

Mussell, H.W. & Strand, L.L. (1977).
Pectic enzymes: involvement in pathogenesis and possible relevance to tolerance and specificity, in *Cell Wall Biochemistry Related to Specificity in Host-Plant Pathogen Interactions*, eds Solheim, B. & Raa, J., pp 31-70. Oslo: Universitetsforlaget.

Mussell, H.W. & Strouse, B. (1972).
Characterisation of two polygalacturonases produced by *Verticillium albo-atrum*. *Can. J. Bot.* **50**, 625-632.

Myers, M.G. & Eberhart, B. (1966).
Regulation of cellulase and cellobiase in *Neurospora crasa*. *Biochim. Biophys. Acta*. **24**, 782-785.

Nagel, C.W. & Anderson, M.M. (1965).
Action of bacterial *trans*-eliminse on normal and unsaturated oligogalacturonic acids by an *endo*-pectic acid *trans*-eliminase. *Arch Biochem. Biophys.* **118**, 590-595.

Nakadai, T., Nasuno, S. & Iguchi, N. (1973).
Purification and properties of leucine aminopeptidase I from *Aspergillus oryzae*. *Agr. Biol. Chem.* **37**, 757-765.

Nasuno, S. & Starr, M.P. (1967).
Polygalacturonic Acid *trans*-eliminase of *Xanthomonas campestris*. Arch. Biochem. Biophys. **104**, 178-185.

Nicholson, R.L., Kúc, J. & Williams, E.B. (1972).
Histochemical demonstration of transitory esterase activity in *Venturia inaequalis*. *Phytopathology* **62**, 1242-1247.

Nothnagel, E., McNeil, M., Albersheim, P. & Dell, A. (1983).
Host pathogen interactions. XXII. A galacturonic acid oligosaccharide from plant cell walls elicits phytoalexins. *Plant Physiol.* **71**, 916-926.

Northcote, D.H. (1972).
Chemistry of the plant cell wall. *Ann. Rev. Plant Physiol.* **23**, 113-132.

Novick, P., Field, C. & Schekman, R. (1980).
Identification of 23 complementation groups required for post-translational events in the yeast secretory pathway. *Cell* **21**, 205-215.

Novick, P. & Schekman, R. (1979).
Secretion and cell-surface growth are blocked in a temparature sensitive mutant of *Saccharomyces cervisiae*. *Proc. Natl. Acad. Sci. USA*. **76**, 1858-1862.

O'Connell, R.J., Bailey, J.A., Vose, I.R. & Lamb, C.J. (1986).
Immunogold labelling of fungal antigens in cells of *Phaseolus vulgaris infected by Colletotrichum lindemuthianum*. *Physiol. Plant Pathol.* **28**, 99-105.

Olutiola, P.O. & Ayres, P.G. (1973).
Utilisation of carbohydrates in *Rhyncosporium secalis*. I. Growth and sporulation on glucose, galactose and galacturonic acid. *Physiol. Plant Pathol.* **29**, 92-96.

O'Neil, M.A. & Selvendran, R.R. (1980a).
lycoproteins from the cell wall of *Phaseolus coccineus*. *Biochem. J.* 187, 53-63.

Paigen, K. & Williams, B. (1970).
Catabolite repression and other control mechanisms in carbohydrate utilisation. *Adv. Microb. Physiol.* **4**, 251-323.

Paoni, N.F. & Arroyo, R.L. (1984).
Improved method for detection of glycosidases in bacterial colonies. *Appl. Environ. Microbiol.* **47**, 208-209.

Partridge, S.M. (1949).
Aniline hydrogen pthalate as a spraying reagent for chromatography of sugars. *Nature* **164**, 443.

Pastan, I. & Perlman, R. (1970).
Cyclic adenosine monophosphate in bacteria. *Science* **169**, 339-344.

Patil, S.S. & Dimond, A.E. (1967).
Inhibition of *Verticillium* polygalacturonase by oxidation products of polyphenols. *Phytopathology* **57**, 492-496.

Pegg, G.P. (1981).
Biochemistry and physiology of pathogenesis. In *Fungal Wilt Diseases of Plants*, eds Mace, M.E., Bell, A.A. & Beckman, C.H., pp 427-521. Academic Press.

Pegg, G.F., Gull, K. & Newsam, R.J. (1976).
Transmission electron microscopy of *Verticillium albo-atrum* hyphae in xylem vessels of tomato plants. *Physiol Plant Pathol.* **8**, 221-224.

Pierepoint, W.S. (1969).
O-quinones formed in plant extracts. Their reaction with amino acids and peptides. *Biochem. J.* **112**, 619-629.

Pierson, C.F., Gothoskar, S.S. Walker, J.C. & Stahman, M.A. (1955).
Histological studies on the role of pectic enzymes in the development of *Fusarium* wilt, symptoms in tomato. *Phytopathology* **45**, 524-527.

Pilnik, W. & Voragen, A.G.J. (1970).
Pectic substances and other uronides. In *Biochemistry of fruits and their products*, ed, Hulme, A.C. , pp 53-87. Academic Press.

Pirt, S.J. (1971).
The diffusion capsule, a novel device for the addition of a solute at a constant rate to a liquid medium. *Biochem. J.* **121**, 293-297.

Plumbley, R.A., & Pitt, D. (1979).
Purification and properties of a pectin-methyl *trans*-eliminase, a phospholipase and two amonopeptidases produced in culture by *Phoma medicaginis var. pinodella*. *Physiol. Plant Pathol.* **14**, 313-328.

Polachek, Y. & Rosenberger, R.F. (1975).
Autolytic enzymes in hyphae of *Aspergillus nidulans*: their action on old and newly formed walls *J. Bacteriol.* **121**, 323-337.

Porter, F.M. (1969).
Protease activity in diseased fruits. *Phytopathology* **56**, 1424-1425.

Pugsley, A.P., & Schwartz, M. (1985).
Export and secretion of proteins by bacteria. *FEMS Microbiology Reviews* 32, 3-38.

Puhalla, J.E. & Bell, A.A. (1981).
Genetics and biochemistry of wilt pathogens. In *Fungal Wilt Diseases of Plants*, eds Mace, M.E., Bell, A.A. & Beckman, C.H., pp 427-521. Academic Press.

Puhalla, J.E., & Howell, C.R. (1975).
Significance of *endo*-polygalacturonase activity to symptom expression of *Verticillium* wilt in cotton, assessed by the use of mutants of *Verticillium dahliae* Kleb. *Physiol. Plant Pathol.* 7 147-152.

Purdy, R.E. & Kolattukudy, P.E. (1975).
Depolymerisation of a hydroxy fatty acid bipolymer, cutin, by an extracellular enzyme from *Fusarium solani f. pisi*. Isolation and some properties of the enzyme. *Arch. Biochem. Biophys.* 169, 61-69.

Quantick, P., Cervone, F. & Wood, R.K.S. (1983).
Isozymes of a polygalacturonate *trans*-eliminase produced by *Erwinia atroseptica* in potato tissue and in liquid culture. *Physiol. Plant Pathol.* 22, 77-86.

Rattigan, A.T. & Ayres, P.G. (1977a).
Pyridine nucleotide linked hexuronic acid hydrogenase activity in mycelium of *Sclerotinia fructigena*. *Trans. Br. mycol. Soc.* 69, 502-503.

Rattigan, A.T. & Ayres, P.G. (1977b).
Uptake of ^{14}C-labelled D-uronic acids by *Sclerotinia fructigena* mycelium. *Trans. Br. mycol. Soc.* 69, 443-449.

Rees, D.A. (1982).
Polysaccharide conformation in solutions and gels-recent results on pectins. *Carbohydr. Polymers* 2, 254-263.

Reese, E.T. (1977).
Degradation of polymeric carbohydrates by microbial enzymes. In *Structure, Biosynthesis and degradation of Wood*, eds, Loewus, F.A & Runeckles V.C., pp 311-367. *Recent Advances in Phytochemistry*. Plenum Press.

Rexanová-Berková, L. & Markovîc, O. (1976).
Pectic enzymes. *Adv. Carbohydrate Biochem.* 33, 323-385.

Rexanová-Berková, L. & Slezarik, A. (1968).
Molecular weight and composition of *Aspergillus niger* endopolygalacturonase. *Collection of Czeckoslovak Chemical Communications* 33, 1965-1967

Rhaese, H.J. & Boetker, N.K. (1973).
The molecular basis of mutagenesis by methyl and ethyl sulphonate. *Eur. J. Biochem.* 32, 166-172.

Ricart, J. & Noat, G. (1986).
Electrostatic effects and the dynamics of enzyme reactions at the surface of plant cells. I. A theory of the ionic control of a complex multi-enzyme system. *Eur. J. Biochem.* 155, 183-190.

Ried, J.L. & Collmer, A. (1985).
Activity stain for rapid characterisation of pectic enzymes in isoelectric focusing and sodium dodecyl sulfate-polyacrylamide gels. *Appl. Environ. Microbiol.* 50, 615-622.

Roberts, D.P., Berman, P.M., Allen, C., Stromberg, V.K., Lacy, G.H. & Mount, M.S. (1986a).
Erwinia carotovora: molecular cloning of a 3.4 kilobase DNA fragment mediating production of pectate lyases. *Can. J. Plant Pathol.* 8, 17-27.

Roberts, D.P., Berman, P.M., Allen, C., Stromberg, V.K., Lacy, G.H. & Mount, M.S. (1986b).
Requirement for two or more *Erwinia carotovora* subsp. *carotovora* pectolytic gene products for maceration of potato tuber tissue by *Escherichia coli*. *J. Bacteriol.* 167, 279-284.

Roeder, D.L. & Collmer, A. (1985a).
Marker-exchange mutagenesis of a pectate lyase isozyme gene in *Erwinia chrysanthemi*. *J. Bacteriol.* 164, 51-56.

Roeder, D.L. & Collmer, A. (1985b).
Marker-exchange mutagenesis of the *pelB* gene in *Erwinia chrysanthemi* CUCPB 1237. Proceedings VI Conference on Plant Pathogenic Bacteria, college Park Maryland, USA.

Roland, J.C. & Vian, B. (1971).
Réactivité du plasmalemme végétal: étude cytochimique. *Protoplasma* 73, 121-137.

Rombouts, F.M. & Pilnick, W. (1980).
Pectic enzymes. In *Microbial enzymes and Transfomation*, ed Rose, A.H., pp 228-283. Academic Press: London.

Rossall, S. & Mansfield, J.W. (1978).
The activity of wyerone acid against *Botrytis* spp. on broad bean leaves (*Vicia faba* L.). *Physiol Plant Pathol.* 16, 369-382.

St. Leger, R.J. (1985).
Cuticle-degrading enzymes of entomopathogenic fungi. Ph.D. Thesis, University of Bath.

St. Leger, R.J., Charnley, A.K. & Cooper, R.M. (1986a).
Cuticle-degrading enzymes of entomopathogenic fungi: synthesis in culture on cuticle. *J. Invertebr. Pathol.* 48, 85-95.

Sargent, C. & Gay, J.L. (1977).
Barley epidermal apoplast structure and modification by powdery mildew contact. *Physiol. Plant Pathol.* 11, 195-205.

Schaible, L. (1941).
Inheritance of resistance to *Verticillium* wilt in a tomato cross. *Phytopathology* 41, 986-990.

Schoedel, C. & Collmer, A. (1986).
Evidence of homology between the pectate lyase-encoding *pel* B and *pel* C genes in *Erwinia chrysanthemi*. *J. Bacter* 167, 117-123.

Scott, S.W. & Fielding, A.H. (1985).
Differences in pectolytic enzyme patterns induced in *Sclerotinia trifoliorum* by different legume host species. Trans. Br. mycol. Soc. 84, 317-324

Selvendran, R.R. (1985).
Developments in the chemistry and biochemistry of pectic and hemicellulosic polymers. *J. Cell Sci. Suppl.* 2, 51-88.

Shaykh, M., Soliday, C. & Kolattukudy, P.E. (1977).
Proof for the production of cutinase by *Fusarium solani pisi*. *Plant Physiol.* 60, 170-172.

Sheiman, M.I., McMillan, J.D., Millar, L. & Chase, T. (1976).
Coordinated action of pectinesterase and polygalacturonate lyase complex of *Clostridium multifermentans*. *Eur. J. Biochem.* 64, 565-572.

Sheppard, D.E. & Englesberg, E. (1967).
Further evidence for positive control of the L-arabinose system by gene *ara* c. *J. Molec. Biol.* 22, 443-454.

Shishiyama, J., Arakai, F. & Akai, S. (1970a).
Studies on cutin-esterase I. Preparation of cutin and it's fatty acid component from tomato peel. *Plant & Cell Physiol.* 11, 323-334.

Shishiyama, J., Arakai, F. & Akai, S. (1970b).
Studies on cutin-esterase II. Characteristics of cutin-esterase from *Botrytis cinerea* and it's activity on tomato-cutin. *Plant & Cell Physiol.* 11, 937-945.

Slots, J. (1981).
Enzymatic characterisation of some oral and non oral Gram-negative bacteria with the Api-Zym system. *J. Clin. Microbiol.* 14, 288-294.

Smith, K.C. (1977).
The Science of Photobiology. Plenum Press: New York.

Stack, J.P., Mount, M.S., Berman, P.M. & Hubbard, J.P. (1980).
Pectic enzyme complex from *Erwinia carotovora*: a model; for degradation and assimilation of host pectic fraction. *Phytopathology* 70, 267-272.

Stanier, R.Y., Adelberg, E.A. & Ingram, J.L. (1976).
General Microbiology. Macmillan Press: London.

Stent, G.S. & Callender, R. (1978).
Molecular Genetics, an introductory narrative. Freeman & Co.

Stevens, B.J.H. & Selvendran, R.R. (1984d).
Structural features of cell-wall polymers of the apple. *Carbohydr. Res.* 135, 155-166.

Stevens, G.J. & Wood, R.K.S. (1974).
Killing of protoplasts by soft-rot bacteria. *Physiol Plant Pathol.* 5, 165-181.

Stewart, A. (1983).
Mechanisms of resistance to Botrytis allii on onion (*Allium cepa* L.).
Ph.D. Thesis, University of Stirling.

Stewart, A. & Mansfield, J.W. (1985a).
Fungal development and plant response in detached onion bulb scales and leaves inoculated with *Botrytis allii*, *B. cinerea*, *B. fabae* and *B. squamosa*. *Plant Pathol.* 33, 401-409.

Strand, L.L. & Mussell, H.W. (1975).
Solubilisation of peroxidase activity from cotton cell walls by endopolygalacturonase. *Phytopathology* 65, 830-831.

Strand, L.L., Rechtoris, C. & Mussell, H. (1976).
Polygalacturonases release cell-wall-bounnd proteins. *Plant Physiol.* 58, 722-725.

Sweetingham, M.W., Cruickshank, R.H. & Wong, D.H. (1986).
Pectic zymograms and taxonomy and pathogenicity of the *Ceratobasidiaceae*. *Trans. Br. mycol. Soc.* 86, 305-311.

Swinburne, T.R. & Corden, M.E. (1969).
A comparison of the polygalacturonase produced *in vivo* and *in vitroi* by *Penicillium expansum*. *J. Gen Microbiol.* 55, 75-87.

Talboys, P.W. (1958).
Some mechanisms contributing to *Verticillium* resistance in the hop root. *Trans. Br. mycol. Soc.* 41, 227-241.

Talboys, P.W. & Busch, L.V. (1970).
Pectic enzymes produced by *Verticillium* species. *Trans. Br. mycol. Soc.* 55, 367-381.

Talmadge, K.W., Keegstra, K., Bauer, W.D. & Albersheim, P. (1973).
The structure of plant cell walls. I. The macromolecular components of the walls of suspension-cultured sycamore cells with a detailed analysis of the pectic polysaccharides. *Plant Physiol.* 51, 158-173.

Tepfer, M. & Taylor, I.E.P. (1981).
The permeability of plant cell walls as measured by gel filtration chromatography. *Science* 213, 761-763.

Thanassoulopoulos, C.C., Biris, D.A. & Tjamos, E.C. (1981).
Occurence of *Verticillium* wilt in weeds in olive orchards. International *Verticillium* Symposium, p 58 (Abstract). Instuto di Patologica Vegetale. University of Bari: Italy.

Thurn, K.K. & Chatterjee, A.K. (1985).
Single-site chromosomal Tn-5 insertions affect the export of pectolytic and cellulolytic enzymes in *Erwinia chrysanthemi* EC-16. *Appl. Environ. Microbiol.* 50, 894-898.

Threlfall, R.J. (1971).
Mutants of *Botrytis allii* Munn. Microgenet. Bull. 33, 4.

Trevelyan, W.E., Procter, D.E. & Harrison, J.S. (1950).
Detection of sugars on paper chromatograms. *Nature* 166, 444-445.

Tribe, H.T. (1955).
Studies in the physiology of parasitism. XIX. On the killing of plant cells by enzymes of *Botrytis cinerea* and *Bacterium aroideae*. *Ann. Botany* 19, 351-368.

Tsyumu, S. (1979).
"Self catabolite repression" of pectate lyase in *Erwinia carotovora*. *J. Bacteriol.* 137, 1035-1036.

Typas, M.A. & Heale, J.B. (1976a).
Heterokaryosis and the role of cytoplasmic inheritance in dark resting structure formation in *Verticillium* spp. *Molec. Gen. Genet.*, **146**, 17-26.

Typas, M.A. & Heale, J.B. (1976b).
Heterokaryosis and the role of cytoplasmic inheritence in dark resting structure formation in *Verticillium* spp. *Molec. Gen. Genet.* **146**, 17-26.

Typas, M.A. & Heale, J.B. (1978).
Heterozygous diploid analyses via the parasexual cycle and a cytoplasmic pattern of inheritance in *Verticillium* spp. *Genet. Res.* **31**, 131-144.

Urbanek, H. & Zalweska-Sobczak, J. (1975).
Polygalacturonase of *Botrytis cinerea* E-200 PERS. *Biochim Biophys Acta* 377, 402-409.

Van der Molen, G.E., Beckman, C.H. & Rodehorst, E. (1977).
Vascular gelation: A general response following infection. *Physiol Plant Pathol.* **60**, 641-646.

Van Gijsegem, F.A., Toussaint, A. & Schoonejans, E. (1985).
In vivo cloning of the pectate lyase and cellulase genes of *Erwinia chrysanthemi*. *EMBO J.* **4**, 787-792.

Verhoeff, K. (1974).
Latent infections by fungi. *Ann. Rev. Phytopath.* **12**, 99-110.

Verhoeff, K. (1980).
The infection process and host-pathogen interactions. In *The Biology of Botrytis*, eds Coley-Smith, W.R., Jarvis, W.R. & Verhoeff, K., pp 153-180. Academic Press.

Verhoeff, K. & Liem, J.I. (1978).
Presence of *endo*-polygalacturonase in
conidia of *Botrytis cinerea* before and during germination *Phytopathol. Z.* **91**, 110-115.

Verhoeff, K. & Warren, M. (1972).
In vitro and *in vivo* production of cell wall degrading enzymes by *Botrytis cinerea* from tomato. *Neth J. Pl. Path.* **78**, 179-185.

Wang, M.C. & Keen, N.T. (1970).
Purification and characterisation of *endo*-polygalacturonase from *Verticillium albo-atrum*. *Arch. Biochem. Biophys.* **141**, 749-757.

Warren, L. (1960).
Thibarbituric acid spray reagent for deoxy sugars and sialic acids. *Nature* **186**, 237.

Vijesundera, R.L.C., Bailey, J.A. & Byrde, R.J.W. (1984).
Production of pectin lyase by *Colletotrichum lindemuthianum* in culture and in infected bean (*Phaseolus vulgaris*) tissue. *J. Gen. Microbiol.* **130**, 285-290.

Wheeler, H. (1977).
Ultrastructure of penetration by *Helminthosprium maydis*. *Physiol. Plant Pathol.* **11**, 171-178.

Williams, W.J. (1979).
Handbook of Anion Determination, pp 62-67. Butterworths.

Willetts, H.J., Byrde, R.J.W., Fielding, A.H. & Wong, A.L. (1977).
The taxonomy of brown rot fungi (*Monilinia* spp.) related to their extracellular cell wall-degrading enzymes. *J. Gen. Microbiol.* **103**, 77-83.

Wood, R.K.S. (1961).
Verticillium wilt of tomatoes - the role of pectic and cellulolytic enzymes. *Ann. Appl. Biol.* **49**, 120-139.

Wood, R.K.S. (1967).
Physiological Plant Pathology. Blackwell Scientific Publications.

Wood, T.M. & McCrae, S.I. (1979).
Synergism between enzymes involved in the solubilisation of native cellulose. In *Advances in Chemistry*, Vol 181. *Hydrolysis of Cellulose: Mechanisms of Enzymatic and Acid Catalysis*, eds Brown, R.D. & Juralsek, L., pp 181-209. American Chemical Society.

Yamazaki, N., Fry, S.C., Darvill, A.G. & Albersheim, P. (1983).
Host pathogen interactions. XXIV. Fragments isolated from suspension-cultured sycamore cell walls inhibit the ability of cells to incorporate ^{14}C-leucine into proteins. *Plant Physiol.* **72**, 864-869.

Yelton, M.M., Hammer, J.E. & Timberlake, W.E. (1984).
Transformation of *Aspergillus nidulans* by using a *trp C* plasmid. *Proc. Natl. Acad. Sci. USA* **81**, 1470-1474.

Yoder, O.C., Weltring, K., Turgeon, B.G. & Van Etten, H.D. (1986).
Technology for molecular cloning of fungal virulence genes. In *Biology and Molecular Biology of Plant Pathogen Interactions*, ed Bailey, J.A. Plenum Press: New York.

Zimmermann, F.K., Schwater, R. & Loer, V. (1966).
Mitotic recombination induced in *Saccharomyces cervisiae* with nitrous acid, diethyl sulphate and carcinogenic alkylating nitrosoamines. *Z. Verebungslehre* **98**, 230-246.

Zink, F.W. (1966).
Studies on the growth rate and nutrient absorbtion of onion. *Hilgardia* **37**, 203-218.

Zink, R.T. & Chatterjee, A.K. (1985).
Cloning and expression in *Escherichia coli* of pectinase genes of *Erwinia carotovora* subsp. *carotovora*. *Appl Environ. Microbiol.* **49**, 714-717.

Zucker, M. & Hankin, L. (1970).
Regulation of pectate lyase synthesis in *Pseudomonas fluorescens* and *Erwinia carotovora*. *J. Bacteriol.* **104**, 13-18.